Radiation-Induced Bowel Injury (RIBI): Exploring potential predictive and prognostic factors and strategies to improve the management of women treated with Pelvic Radiation for Cervical and Endometrial Cancers.

Cancer Survivorship: Improving Quality of Life after Treatment for Gynaecological Cancers

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DECLARATION

Statement of Originality.

All work presented in this thesis was undertaken by myself unless otherwise stated.

Abstract

Introduction: The true incidence of radiation toxicity to the bowel remains unknown; in the UK, it has been reported that about 90% of patients who receive pelvic radiotherapy will have some change in their bowel function, and in up to 50% this affects their quality of life significantly (Andreyev, 2007). It is unclear why some cancer survivors develop significant symptoms that arise as a result of multiple functional, structural and physiological deficiencies related to radiation injury. **Aims**: There is a need to identify tissue specific biomarkers of normal tissue injury and identify those patients who might be at risk of severe injury to the bowel. In this thesis, I sought to investigate the true incidence and presentation of RIBI in a London Cancer Centre. I then developed a template for a scoring model to explore how reporting of symptoms in the clinical setting might be improved. After investigating the use of cell-cycle markers as a marker of (chemo)-radiosensitivity, I then utilised these markers in colonic crypt cells to attempt to link the proliferative status after exposure to radiation to symptom presentation and severity. **Methods and Results**: A retrospective cohort study revealed 152 women treated for cervical and endometrial cancer with symptoms of RIBI, which were clustered into 3 groups using factor analysis. Exploratory and Confirmatory Factor Analysis was used to test a novel scoring model template. Immunostaining in 35 cervical tumour samples with the cell-cycle markers Mcm2, Geminin, and Ki67 did not find expression of these markers were linked to (chemo)-radiosensitivity and tumour response. These markers were used to assess proliferation in colo-rectal crypt cells and showed decreased expression in all layers suggesting a loss of proliferative capacity after radiation. **Conclusions**: Young patients with cervical cancer are more likely to develop significant symptoms of RIBI. Our simple scoring tool validated on a prospective cohort could provide invaluable data to improve management of women with bowel symptoms after radiation. Further work exploring proliferation in colonic crypt cells after radiation exposure could identify women at greater risk of radiation injury.

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List of Abbreviations

ABO	Acute Bowel Obstruction
AUC	Area Under the Curve
ATM	Ataxia Telangiectasia Mutated protein
ATR	ATM- and Rad3-related protein kinase
Cdt1	Chromosome licensing and DNA replication factor 1
Cdc6	Cell division control protein 6 homologue
CRT	Chemo-radiation
CI	Confidence Interval
CTV	Clinical Target Volume
CFI	Comparative Fit Index
CFA	Confirmatory Factor Analysis
CTCAE	Common Terminology Criteria for Adverse Events
CT	Computerised Tomography
DFS	Disease-Free Survival
EBRT	External Beam Radiotherapy
EUA	Examination Under Anaesthesia
EORTC	European Organization for Research and Treatment of Cancer
EFA	Exploratory Factor Analysis
FIGO	International Federation of Obstetrics & Gynaecology
Gy	Gray
HDR	High-Dose Rate
HTPN	Home Parenteral Nutrition
IBS	Irritable Bowel Syndrome
ICB	Intra-Cavity Brachytherapy
IMRT	Intensity Modulated Radiotherapy
IBDQ-B	Inflammatory Bowel Disease Questionnaire - Bowel Subset
MCM	Minichromosome maintenance
MRI	Magnetic Resonance Imaging
OR	Odds Ratio
ORC	Origin Recognition Complex
OS	Overall Survival
PALN	Para-aortic Lymph Nodes
PRD	Pelvic Radiation Disease
PCA	Principal Components Analysis
PTV	Planning Target Volume
QoL	Quality of Life
RIBI	Radiation-induced Bowel Injury
ROC	Receiver Operating Characteristics
RTOG	Radiation Therapy Oncology Group
RLFs	Replication Licensing Factors
RMSEA	Root Mean Square Error of Approximation
SABO	Sub-Acute Bowel Obstruction
SeCHAT	Tauroselcholic[75selenium]acid
SOMA	Subjective, Objective, Management and Analytic
TAH	Total Abdominal Hysterectomy
TGF-β	Transforming Growth Factor- β
TLH/BSO	Total Laparoscopic Hysterectomy and Bilateral Salpingo-oophorectomy
UC	Ulcerative Colitis

Publications

Oral Presentations

RCOG/ENTRIGO Translational Research Workshop – September 2012

Do cell-cycle Phase-Specific Markers Predict Disease Grade, Stage and Outcome in Cervical Carcinoma?

Kuku S, Proctor I, Loddo M, Kadalayil L, KhoshZaban M, Ledermann J, McCormack M.

Peer-Review Journals

Do cell-cycle Phase-Specific Markers Predict Disease Grade, Stage and Outcome in Cervical Carcinoma?

Kuku S, Proctor I, Loddo M, Kadalayil L, KhoshZaban M, Ledermann J, McCormack M.

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Do cell-cycle Phase-Specific Markers Predict Disease Grade, Stage and Outcome in Cervical Carcinoma?

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1. A Clinical Score To Predict Severity Of Radiation-induced Bowel Injury (RIBI): Improving Reporting Of Symptoms After Pelvic Radiation. Kuku S, Fragkos C, McCormack M, Forbes A.

2. Can Cell Cycle Markers Predict Severity In Patients With Symptoms Of Radiation-induced Bowel Injury (RIBI) After Treatment For Cervical And Endometrial Cancers? Kuku S, Proctor I, Fragkos C, McCormack M, Forbes A.

Part I Radiation Induced Bowel Injury

Chapter One Introduction

1.1 History of Radiation use in Cancer

In 1895, the discovery of ionising radiation by Wilhelm Roentgen changed modern medicine. By 1896, Emil Grubbe, recognised as the world's first Radiation Oncologist had made the first attempt to treat breast cancer with radiation therapy and soon after in 1897, the first patients with radiation-induced bowel toxicity symptoms were described (Walsh, 1897). Claude Regaud, a Professor at the Radium institute of Paris, recognised quickly that treatment was better tolerated and more effective if delivered slowly with modest doses per day over several weeks. This technique, still known today as fractionation still remains the main principle behind radiation delivery in Radiation Oncology.

Early X-ray machines had numerous limitations and were unable to produce high energy, deeply penetrating beams, thus making it difficult to treat deep-seated tumours (especially in the pelvis) without excessive skin reactions. Early advocates of radiation therapy began to rely on the placement of radioactive sources in close proximity or even within the tumour, a technique still known today as Brachytherapy. In many pelvic tumours, most notably cervical and endometrial cancers in women, brachytherapy became the mainstay of curative treatment regimes (and mostly remains so in the 21st century). The Radium Institute of London opened in 1911, and intracavity treatment began on a large scale. By the early 1920s, the combination of both external beam deep x-ray treatment and brachytherapy had become the standard with a reported 5-year survival of 45% (Phillips, 1944). Improvements in treatment delivery have occurred, first with the introduction of supervoltage radiotherapy in the 1940s and then subsequently with the linear accelerator in the 1960s. Attempts at radio-sensitisaton using agents such as hyperbaric oxygen in the 1970s and early 1980s were disappointing.

1.2 Early Retrospective Study – History of Radiation Toxicity

The early 20th century brought advances mainly in optimising treatment doses and delivery, as well as in planning treatment, through advances in radiation physics and oncology. A retrospective review (DeCosse et al, 1969) of radiation-induced toxicity at University Hospitals Cleveland of 100 patients treated for pelvic malignancies between 1922 and 1968 was published in 1969. Gastrointestinal complications following radiation for cervical cancer was reported in 11.6% of patients. In this cohort bowel injuries were multiple and defined as; 'proctitis': n=44; 'rectal ulcer': n=10; 'stricture': n=19; 'rectovaginal fistula': n=29; 'colitits': n=17; 'ileitis' or 'jejunitis': n=25; rectal 'polyps': n=1; 'radiation-induced rectal carcinoma': n=1. The majority (95%) of patients in this early study were women (carcinoma of cervix: n=75; carcinoma of uterus: n=9; bladder carcinoma: n=2; vagina: n=5; prostate: n=1). The average age of patients was 52 years range (1-77). Previous 'supra-cervical' hysterectomy had been performed in 14 women, adnexal surgery in 12, and appendicectomy in 12. Over 50% of patients (57/100) had a significant medical history; hypertension in 39, arteriosclerotic heart disease in 15, diabetes in 9, 'intestinal' disease in 6, previous cancer in 3, 'rectal disease' in 3, previous tuberculosis infection in 2, and syphilis in 2. Ten (10) women had suffered previous pelvic inflammatory disease (PID). In 78% of patients treatment was delivered with curative intent. External beam radiation alone was administered in 17, radium alone in 6, and the remaining patients had a combination of both. Sixteen percent (16/100) of the patients had surgery combined with radiotherapy. In this study death was attributed to radiation-induced bowel injury in 22/100 (22%) patients (mean follow-up 7 years); 28/100 patients died of cancer-related causes.

1.2.1 Early experience of Radiation Toxicity after treatment for Cervical Cancer

The cervical cancer patients with reported symptoms of toxicity in this study (DeCosse et al, 1969) were compared to 227 cervical cancer patients treated during the same period with no gastrointestinal symptoms. It is unlikely that a true comparison could have been made between these two groups given the study was conducted over a 40 year period. It

is also important to note that treatment doses and delivery varied significantly over this period. Nevertheless, the group found a significant relationship between having a history of hypertension and reporting radiation-induced bowel symptoms ($p<0.025$). Pre-radiation supra-cervial hysterectomy was also found to be significantly associated with bowel toxicity ($p<0.025$). Of the 75 patients with cervical cancer, 67 were treated with external pelvic radiation (EBRT).

External beam therapy was considered 'high-dose' if the dose to the mid-plane of the pelvis was >9,000 rads. Intra-cavity radium was employed in 67/75 (89%) women with cervical cancer. Seven patients, (29.1%) with non-rigid radium applications, and 8 (18.6%), with Ernst radium application were considered to have high radium dosage. Thus, 20.0% of all patients with cervical carcinoma received radium applications in excess of 7,000 rads for one application, or 9,000 rads for two applications.

1.2.2 Radiation-Induced Bowel Injury as reported in 1969

Patients with rectal injury in this study (DeCosse et al, 1969) were classified into two groups for analysis. The first group of patients (n=52) presented with *proctitis, rectal ulcer and stenosis*, mostly within the first year after radiation therapy; 14 patients presented two or more years after completion of treatment. Symptoms of diarrhoea, bleeding and tenesmus were not found to correlate well with the extent of rectal pathology on histology. The presence of crampy abdominal pain was unusual in pure rectal injury and generally could be related to more distal bowel injury. Radiation-induced rectal ulcers were usually reported between 4 and 12 months after start of treatment and were usually single, less than 4cm in diameter and seen on the anterior rectal wall at the level of the cervix. The second group of patients described consisted of 29 patients with confirmed recto-vaginal fistulas. The average interval from completion of radiation therapy to presentation was 22 months (range: 3 months -12 years). Interestingly, it was reported that prior to developing fistulas these patients had reported symptoms similar to the first group but generally more severe in nature.

In this series, 34 patients were reported to have non-rectal bowel injury: small bowel in 17, colon in 9, and to both small and large bowel in 8. Twenty-five (n= 25) of these patients were classified as having *ileitis/jejunitis*. In 21 of these 25 patients the diagnosis was confirmed with a histopathological examination of operative biopsy samples or autopsy findings. In the remaining 4 patients, barium examination of the small bowel (indicated by presenting symptoms) demonstrated radiation injury. Thirteen (13/34) patients also had rectal injury and 15 had associated urinary tract injury. Patients with small bowel injury were reported to have presented with 'ileitis' or symptoms of partial to complete (small bowel), intestinal ischaemia or necrosis, and less commonly, with an enteric fistula.

The presenting findings of colon injury were reported as 'colitis', stenosis or obstruction, (usually sigmoid), necrosis and a sigmoid vaginal fistula. In the small bowel injury group, in patients presenting with symptoms of acute bowel obstruction or perforation, the interval from radiation to presentation was on average 6.5 years (range 1 month – 31 years). Sixteen patients in this study required surgery for intestinal resection due to complications from radiation-induced injury; bowel perforation, bowel obstruction and vesico-vaginal fistula in one patient. Of the patients who required surgical intervention, 2 patients died during the first 30 days after bowel resection, whilst 3 others died within the 2 years following surgical management of the radiation-induced bowel injury.

This early study, although retrospective, highlighted the multiple and complex ways in which radiation damage to the gastrointestinal tract can present, and the high morbidity related to treatment. The overall toxicity rate was reported at 11.6%, comparable to another study (Powel-Smith, 1965) published in the same decade.

1.3 Radiation in the Modern Era

Since DeCosse and his group published the above study in 1969, with similar results the use of advanced, highly effective radiotherapy techniques and more targeted treatment has evolved. Despite these advances in treatment delivery techniques, radiation toxicity to surrounding healthy normal tissue remains a barrier to achieving better cancer cure

rates (Andreyev, 2007a,b). The number of cancer survivors has increased since the 60s and so too has the number of patients reporting and presenting with symptoms related to radiation injury of bowel, bladder and other tissues.

Much of the technological progress in recent decades has been due to computer and imaging advances, in particular axial-imaging methods and three-dimensional treatment planning. The standard for radiation oncology centres is now to perform computed tomography (CT) - based imaging for treatment planning, with treatment delivery advances combining computer algorithms and software packages, which optimise the number, shapes and intensities of beams. Known as three-dimensional (3D) conformal radiotherapy, this technique allows far more effective coverage of tumours whilst better protecting normal adjacent organs. Modern developments in cellular and molecular biology continue to attempt to find new designs for targeted delivery. A discovery known as the 'bystander' effect, an intercellular signalling pathway described by several authors (Mothersill et al, 2001; Turesson et al, 2003) describes the intercellular signalling pathways whereby irradiated cells exert effects on neighbouring cells. It emphasizes the need to consider the entire tumour microenvironment within modern studies of radiation effects.

1.4 Incidence of Cervical and Endometrial Cancer

Cervical Cancer remains a leading cause of deaths worldwide, and remains the 3rd most common cancer in women and the 12th in the UK, accounting for about 2% of all new cases of cancer in women (Cancer Research UK, 2010). Most women present with locally advanced disease and radiotherapy remains an integral part of standard treatment. Concomitant Chemo-radiation (CRT) - radical radiotherapy (external beam radiotherapy (EBRT) plus intra-cavity brachytherapy (ICB)) together with weekly chemotherapy has improved survival rates (Vale et al, 2010), however toxicity still remains a major issue affecting survivorship and uncomplicated cure rates.

Endometrial cancer remains the most common gynaecological malignancy in the western world, and the 4th most common cancer in women. In 2005-2009, 77.3% of women in the UK survived for 5 years or more (Cancer Research UK, 2010). Endometrial cancer, locally advanced, with high-risk prognostic features, is usually treated with primary surgery (total abdominal hysterectomy (TAH) and bilateral salping-oophorectomy (BSO)) followed by sequential combined adjuvant chemotherapy followed by external beam radiotherapy and vaginal vault brachytherapy (VVB). Patients with 'high-risk' prognostic indicators for locally advanced disease and loco-regional (pelvic) recurrence are usually offered radiotherapy alone without chemotherapy.

1.5 Radiation doses and delivery in Cervical and Endometrial Cancer

Radiation toxicity has been reported in doses as low as 5-12 (Gray) Gy but usually occurs in patients exposed to higher doses (Theis et al, 2010). Pelvic radiotherapy for cervical and endometrial cancer is delivered according to national guidelines. Various dosimetric studies (Leschert, 1995; Roeske et al, 1997) over the last few decades have investigated the effects of radiation on tumour control whilst measuring for toxicity. Roeske et al, (1997) investigated the correlation between treatment and dosimetric factors with the risk of late rectal sequealae in 183 patients treated with radiation therapy for cervical carcinoma. All patients received a combination of EBRT and ICB. The median EBRT dose was 45Gy, delivered in daily doses of 1.6-2.0Gy per fraction. Higher doses were given to patients with locally advanced disease, ranging from 75-85Gy to 80-90Gy in patients with larger lesions. Additional parametrial and para-aortic radiation boosts were also given to patients with advanced disease.

Twenty-eight (15.3%) patients developed late rectal sequelae (n=12; Grade 3 toxicity). Patients with diabetes ($p=0.03$), and those who received conventional EBRT doses >50Gy ($p=0.03$) were found to be significantly associated with the risk of rectal toxicity symptoms on multivariate analysis. A defined threshold above which rectal sequelae were more common was identified over the ranges of doses evaluated, using a linear-quadratic model. This threshold was 87% at a total rectal dose of 60Gy and decreased to

60% at a rectal dose of 80Gy. Figure 1.1 shows the dose-response curve of complication probability, with a clear increase in toxicity over 60Gys.

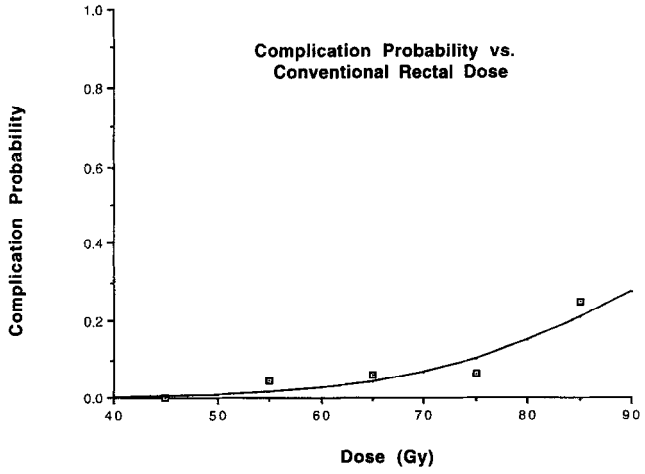


Figure 1.1 Dose (D)-Response curve analysis (Roeske et al, 1997). Plot of the probability of developing rectal sequelae as a function of total rectal dose (maxCRD). Data points represent probabilities determined from index study). The smooth curve represents the maximum likelihood estimate of the logistic function $p(D) = 1/[1 + (TD_{50}/D)^k$; where k=6.6 and TD50 = 105 Grays

The current standard for radical treatment of endometrial cancer is a total dose of 45Gy in 25 daily fractions over 5 weeks in a single phase. Radiotherapy is delivered after 3-dimensional planning with data from computerised tomography (CT) scans. Tolerance dose limits for rectum is limited to <70Gy, whilst the bladder dose limit for the entire course is set at <60Gy. All patients also receive High-Dose Rate (HDR) vaginal vault brachytherapy (VVB) with full insertion of a vaginal applicator; 12Gy in 2 fractions to 0.5cm from the surface of the applicator.

Conventional "box" technique delivers external beam radiotherapy by using bony landmarks to define the target volume for pelvic radiotherapy. Treatment is delivered

either with anterior and posterior fields or with a 4-field box technique, which reduces the volume of the small bowel in the treated volume (Gallagher et al, 1986). Studies assessing coverage of the uterus and cervix have reported that a field border placed in the middle of the symphysis pubis is inadequate for 50-60% of the patients (Bonin et al, 1996), however conventional radiotherapy continues to be used worldwide. In a retrospective review of endometrial cancer (Greven et al, 1991), the incidence of late complications using a 4-field box technique was reduced from 23% to 4% with the use of conformal radiotherapy.

Radiotherapy planning has been improved in the 21st century by the integration of computed tomographic (CT) imaging, allowing the dose of radiation to match or conform to the outline of the target. This shaping of the radiation fields is known as Conformal Radiotherapy and would now be considered best practice in pelvic radiotherapy treatment. Though the fields for conformal radiotherapy are usually larger than those used for the conventional box technique, the individualised shaping of the fields allows more shielding of the organs at risk. Conformal radiotherapy can reduce the mean volume receiving at least 90% of the prescribed dose (V90) by 23%, 4%, 18%, and 11% for the bladder, the rectum, the small bowel, and the large bowel respectively.

In cervical cancer, the outcome of radiotherapy-related treatment changed significantly in 1999 with the National Cancer Institute recommendation (NCI, 1999) based on 5 studies, recommending the use of chemotherapy with radiotherapy. This changed the standard of care overnight. Radical radiotherapy for cervical cancer involves delivery of a total dose of 50.4Gy in 28 daily fractions over 5.5 weeks delivered in a single phase with concomitant weekly chemotherapy (Cisplatin 40mg/m2) weekly for a maximum of 6 weeks during radiotherapy.

1.6 Incidence of Radiation-Induced Injury after treatment of Cervical and Endometrial Cancer; the extent of the clinical problem.

The true incidence of radiation toxicity to the bowel remains unknown; symptoms are generally under-reported, whilst studies in the published literature are mostly incomparable. In the UK, it has been reported that about 90% (Andreyev, 2007a) of patients who receive radiotherapy will have some change in their bowel function, and in up to 50% this affects their quality of life significantly (Andreyev, 2007b). Regardless of modern radiotherapy techniques, the incidence of severe toxicity after (chemo)-radiation for cervical cancer remains about 10% (Vale et al, 2010). A significant proportion of these patients is young and though cured of cancer, are burdened with symptoms of radiation-induced bowel injury (RIBI) which adversely affects their activities of daily living and adds increasing costs to healthcare (Andreyev, 2005).

(Denton et al, 2002) published the results of audit of the prevalence of late severe complications at 5 years following radiotherapy for cervical carcinoma using retrospectively collected morbidity data as an indicator of quality of care. Only severe toxicity (grade 3 and 4) according to the Franco-Italian Glossary (Sinistrero et al, 1993) was recorded. Data was reported from 1558 cases of cervical cancer treated with radical intent in 1993 drawn from 53 of 55 UK treatment centres. In all the centres but one, morbidity was graded retrospectively at the time of the audit. Surgery was combined with radiotherapy in 30.4%, and 6.7% of patients received chemotherapy as part of primary treatment. The 5-year survival of the entire cohort was 47%, and 40% for the subset selected to have radiotherapy alone. The prevalence of late severe morbidity was 6.1%. Variations in treatment technique and administration was observed between 62% of the centres.

"The absence of defined diagnostic criteria for late radiation complications and the multitude of grading systems complicate retrospective reporting although the grade of measurable tissue damage may or may not translate into a corresponding score from the patients perspective. All these discrepancies also need to be recorded if we are to concentrate on measuring the effects that actually matter to patients".

Numerous authors in both retrospective and prospective studies have documented the range in reported incidence of late complications in patients treated with radiation therapy for cervical and endometrial cancers (Andreyev, 2007). Eifel et al, (1995) reported major complication rates at 3 and 5 years of 7.7% and 9.3%, in a retrospective study of 1784 patients with FIGO stage 1B cervical cancer treated with initial radiation between 1960 and 1989. Complication rates were calculated actuarially; at 5 years there was a small but continuous risk of approximately 0.34% a year, with an overall actuarial risk of having had a major complication of 14.4% at 20 years. A recent national survey (Henson et al, 2012) of practice and opinions of clinical oncologists in the UK revealed most oncologists estimate up to 24% of patients at 1 year have bowel symptoms following pelvic radiotherapy. Clinical oncologists continue to recognise late-onset bowel dysfunction after pelvic radiotherapy as a significant problem.

1.7 Radiation Injury to the Bowel -Natural History and Mechanism

1.7.1 Biological Effect of Radiation

The lethal cellular effects of radiotherapy are mediated by DNA damage within tumour cells. Cells undergo a critical period after irradiation that determines their fate: cell death, repaired damage, or continued growth and division without complete repair. On a molecular level, radiation damage initiates very complex signalling cascades that results in a variety of responses including cell cycle arrest, induction of stress response genes, DNA repair, and apoptosis (Connell et al, 2007). The signalling proteins ATM and ATR have a central role in these responses (Fig. 1.2).

Radiation causes cell death through free radicals, which subsequently stimulate single or double-stranded DNA breaks. Electromagnetic waves produce biologic effects on tissues by the production of electrons after their interaction with normal tissues. These electrons induce the formation of hydroxyl and other free radicals. Cellular radiosensitivity is

dependent on the cell cycle. Cells are most vulnerable to the killing effect of radiation during the G2-M phases of the cell-cycle (M- Mitosis). Rapidly proliferating tissues such as intestinal crypt cells are therefore particularly sensitive to radiation. Stem cells within the crypts are particularly at risk and damage to and death of these epithelial cells leads to reduction in differentiated epithelial cells and consequently, a loss of mucosal integrity (Bismar et al, 2002; McGovern, 2005; MacNaughton, 2000).

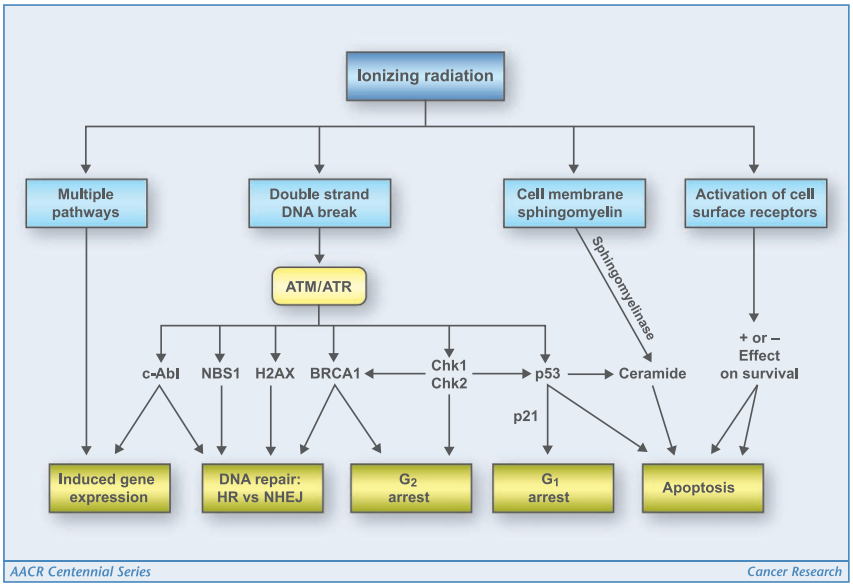


Figure 1.2. A simplified overview of some of the cellular pathways involved in response to ionizing radiation (Connell et al, 2009)

1.7.2 Normal tissue responses to radiotherapy

Radiotherapeutic injury to 'normal' tissue that is included in the radiation field is a complex process that involves multiple responses as a result of repetitive injury during a course of treatment involving a *repetitive* series of exposure to radiation. Whilst the first response to injury involves 'wound healing', the second set of responses perpetuates a cascade of cellular and extracellular responses that includes endothelial cell change and

activation of the coagulation system, inflammation, epithelial regeneration, granulation tissue formation, tissue restitution and remodelling. Figure 1.3 shows the sequence of events involving both the immune system and the microvascular endothelium in acute phase with ongoing responses that are presumed to be responsible for progression of injury over a long period of time (Denham et al, 2002). Healthy tissue toxicity remains the single most important radiation-dose-limiting factor and obstacle to improving cancer cure rates. During radiotherapy of pelvic tumours, parts of the small and large bowel (and bladder) are included in the treatment field and thus the bowel remains at risk, regardless of improvements in treatment delivery techniques.

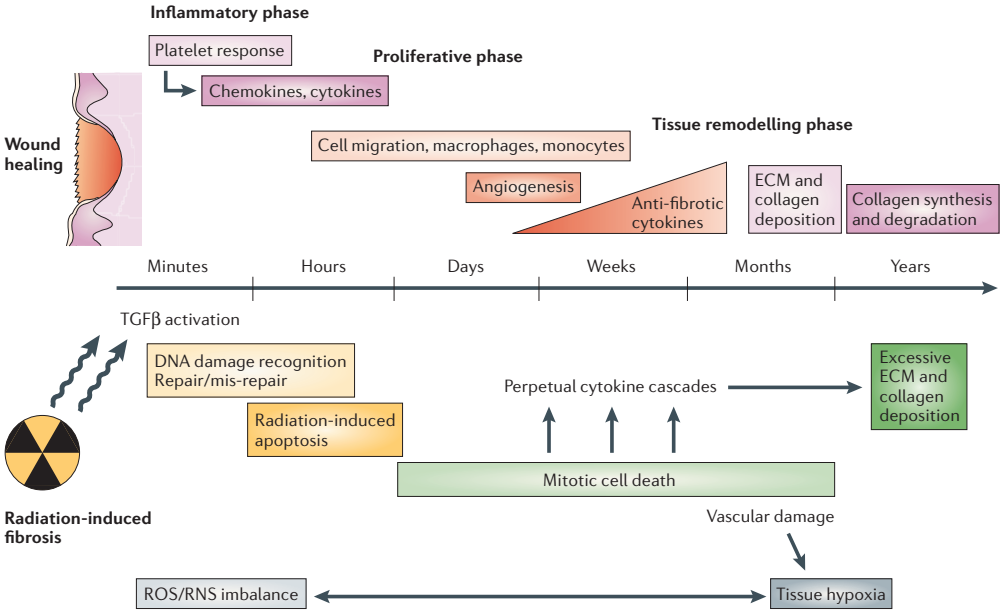


Figure 1.3. Phases of normal wound healing progressing to radiation-induced fibrosis over time (Bentzen MS 2006). Timeline illustrating normal 'wound' healing after radiation trauma. This response to tissue injury is precisely orchestrated and it is probable that continued interference with the normal control of wound healing that leads to excessive deposition of extracellular matrix (ECM) and collagen that is characteristic of radiation fibrosis. ROS, reactive oxygen species; RNS, reactive nitrogen species; TGF-β, transforming growth factor- β.

1.7.3 Molecular Biology of Radiation Effect on Intestines

Under normal physiological conditions, both small intestinal and colonic epithelium have a low rate of spontaneous apoptosis. In the colon, the apoptotic rate is very low because of the presence of *bcl-2*, which protects the cells from programmed cell death. In animal experiments, a rapid increase in the rate of apoptosis of the intestinal crypts was seen when the animals were exposed to low-dose radiation 1-5 centigrays (cGy). The rate of apopotosis was dose dependent and reached a plateau at 1 Gray (Gy). Apopotosis (programmed cell death) induced by radiation is dependent on the presence of p53 in the stem cell (Clarke et al, 1994). Small intestinal cells were most sensitive to radiation compared with stem cells in the colon and rectum because of the presence of bcl-2 in the latter. The 5% risk of complications at 5 years was estimated at a dose level of 45-50Gy for the small intestines and 60-65Gy for the colorectal mucosa (Cohen and Creditor, 1983).

Ionizing radiation also activates the translation of the gene coding for transforming growth factor β (TGF-β). TGF-β is a multifunctional peptide growth factor, classed as a cytokine and growth factor, with a wide range of effects on growth, differentiation, extracellular matrix deposition, and immune response. It is ubiquitous and exists in multiple tissues. There are 3 known isoforms -1, -2, -3. TGF-β-1 is implicated mainly in the pathogenesis of fibrosis by stimulating the expression of collagen and fibronectin genes and the chemostaxis of fibroblasts.

Allgood et al (1996), in a study investigating the influence of timing of concomitant daily boost on the development of intestinal radiation injury in a rat model, observed that radiation injury was significantly more severe when early concomitant (second daily) boosts alone were received earlier (first 6 days) of a 12 week course compared to late boosts (last 6 days). Both the incidence of complications and the degree of histopathologic injury were reduced in the early boost group. Relative collagen content of irradiated intestine was significantly increased in the early boost group compared to the late boost group at 2 weeks and 26 weeks following irradiation. Irradiated intestine in the early boost group exhibited decreased labelling index at 2 weeks whereas irradiated intestine in the late boost group had normal labelling index and increased total crypt

cellularity at 2 weeks. This study, amongst others explains the basis to the evidence for concomitant boosts to be given towards the end of the radiation schedule to minimize the risk of subsequent complications. Normal 'healthy' tissue radiated at the end of a course of treatment differs in repair status at the beginning of the course (Denham et al, 2002).

Cell cycle checkpoints contribute to survival after exposure to ionizing radiation by arresting the cell cycle and permitting repair after an interval. This '*compensatory proliferation*' occurs later in a course of radiotherapy than at the start. Radiation leads to a dose-dependent loss of functional cells through their mitotic death, both immediately after exposure and during the subsequent compensatory phase, resulting in accelerated proliferation as an expression of radiation damage. Consequently, the more severe damage following larger doses of radiation is seen earlier during a course of radiation treatment, than the comparatively milder damage produced with smaller doses of radiation.

1.7.4 Acute Radiation Injury

Acute Radiation Toxicity

Acute or 'early' radiation toxicity is injury which manifests within days of commencing a course of radiotherapy usually occurring within 3 months of radiation therapy and affecting quality of life at the time of treatment. Early intestinal injury is a direct result of cell death in the rapidly proliferating crypt epithelium and an acute inflammatory reaction in the lamina propria. Insufficient replacement of the villus epithelium and breakdown of the mucosal barrier subsequently manifests as symptoms following crypt cell death. Symptoms of acute bowel toxicity include nausea, abdominal pain and cramps, diarrhoea and fatigue. Approximately 60-80% of patients will experience symptoms of acute toxicity (Andreyev, 2007b). Nausea usually occurs almost immediately, while diarrhoea and abdominal pain manifest 2-3 weeks into treatment.

Acute symptoms will usually resolve within 1-3 months of completing treatment (Andreyev et al, 2011).

A prospective study (Hovdenak et al, 2000) examined the sequential development and associations of clinical, endoscopic, and histopathological rectal toxicity during ongoing radiation therapy. Thirty-three (33) patients with pelvic carcinomas (prostate, bladder, and endometrium (n=2)) were assessed before radiation, after 2 weeks of treatment, and towards the end of the treatment course (6 weeks). Rigid sigmoidscopy was performed, and 2-3 forceps biopsies were obtained from the posterior rectal wall, 10cm from the anal verge. Symptoms of acute toxicity were recorded, and endoscopic changes graded. Histological changes in the surface epithelium, glandular layer, and lamina propris were assessed using an ad hoc scoring system, macrophage accumulation was evaluated in anti-CD68 stained sections.

The prevalence of symptoms of acute toxicity increased from baseline to 2 weeks and from 2 to 6 weeks ($p<0.001$). Sixty-one (61%) and 86% of patients had symptoms recorded on questionnaire at 2 and 6 weeks respectively. Pre-treatment endoscopy and biopsies were found to be unremarkable. In contrast, endoscopic changes were maximal at 2 weeks. Biopsies obtained during treatment demonstrated: atrophy of the surface epithelium, acute cryptits, crypt abscesses, crypt distortion and atrophy and stromal inflammation. Histological changes particularly those within the surface epithelium were consistently more pronounced at 2 weeks than they were at 6 weeks; no association was found between endoscopic pathology and individual clinical symptoms at 6 weeks.

1.7.5 Chronic Radiation Injury

Radiation-Induced Bowel Injury (RIBI) is classified as delayed or chronic ‘enteropathy’ when it occurs over 3 months after radiotherapy. Pelvic Radiation Disease (PRD), delayed ‘radiation enteropathy’ or chronic RIBI is usually progressive and has major

implications for cancer survivors and can lead to substantial long term morbidity and mortality if not managed appropriately. The incidence of severe late toxicity (Radiation Therapy Oncology Group – RTOG Grade 3 – 4) has decreased in the last few decades due to improvements in treatment delivery techniques. Regardless, studies suggest that up to half of patients will still suffer symptoms of chronic RIBI (Hauer-Jensen et al, 2014). The incidence of severe toxicity after (chemo)-radiotherapy of cervical cancer remains around 10% (Vale et al, 2010).

The prevalence of chronic radiation enteropathy following radiotherapy for cervical and endometrial cancer and its impact on quality of life was reported in 117 women (Abayomi et al, 2009) who were asked to complete a validated questionnaire exploring bowel problems and quality of life. Responses were scored and compared to scores for women with known faecal incontinence (Bugg et al, 2001) with a condition-specific health-related questionnaire for the assessment of women with anal incontinence. Forty-seven percent (47%) of women gained scores indicative of 'CRE'- chronic radiation enteritis. Younger women ($p<0.001$) and women with cervical cancer ($p<0.05$) were more likely to score for CRE. No significant relationship was observed between score and either radiotherapy dose or stage of cancer.

1.8 Clinical Features and Diagnosis

The main clinical features of chronic RIBI can develop before acute symptoms subside are known to be related to multiple factors including altered intestinal transit, nutrient malabsorption, and gut dysmotility (Husebye et al, 1994; Andreyev, 2007b). As the injury to bowel may be in multiple sites, the range of symptoms patients can present with is vast, ranging from ***abdominal pain***, ***vomiting***, ***diarrhoea*** and ***faecal urgency***, to ***rectal bleeding***, ***per-rectal mucus*** and ***flatulence*** (Andreyev, 2007b). It is well recognised that

symptom aetiology within any one patient can be multi-factorial, with more than half of patients having more than one cause for their symptoms (Andreyev et al, 2005).

Chronic diarrhoea may be secondary to *malabsorption syndromes* (bile salts, carbohydrates), *small bowel bacterial overgrowth*, *new colonic neoplasia*, or *new inflammatory bowel disease*. Complications of chronic progressive injury such as entero-enteric fistula can also contribute to diarrhoea and increase in bowel frequency. Abdominal pain and vomiting can occur as part of acute or sub-acute bowel obstruction or may be related to radiation-induced small (or large) bowel fistulas. Weight-loss, and non-specific symptoms like fatigue and lethargy can also occur (Theis et al, 2010).

Denham et al, (1999) investigated the significance of the various late rectal symptoms that appear after radical prostatic irradiation. Patients with prostate cancer treated between 1987 and 1994 were recalled for examination and asked to complete a detailed questionnaire concerning late radiation-induced symptoms and the effects on their normal daily life. The incidence of acute 'proctitis' symptoms occurring during therapy and the late symptoms recorded were assessed, as well the relationship between late symptoms and late EORTC/RTOG score and impact on normal daily life. The presence of symptoms of acute proctitis was the only factor to predict the presence of late symptoms and late EORTC/RTOG score in this series (odds ratio (OR): 1.7-2.57, p=0.009-0.0007).

Cluster analysis revealed the presence of five subgroups of patients with varying permutations of different late rectal symptoms, including one group with minimal symptoms ($p<0.0001$). While bleeding and rectal discharge were the major contributors to late EORTC/RTOG score ($p<0.0001$ and 0.04), *faecal urgency*, and *bleeding* were found to be the most important factors to impact on normal life ($p<0.0001$ and $p<0.0003$). A relatively low concordance was found between late EORTC/RTOG score and the patients' self-assessment of the effect of their symptoms on their normal daily lives. Some late symptoms including bleeding and rectal discharge become less prevalent after 3 years of follow-up with a resulting improvement in EORTC/RTOG score. Other studies have shown faecal urgency to be at the 'core' of symptoms in patients with significant symptoms of RIBI (Routledge et al, 2003; Putta and Andreyev, 2005; Capp et al, 2009).

In one of the earliest prospective studies in the published literature (Yeoh et al, 1993), the authors evaluated both the short and long-term effects of radiation therapy on gastrointestinal function in 27 patients with potentially curable disease (female: 23; male: 4) before commencement of radiation, during, and 6-8 weeks, 12-16 weeks and 1-2 years after completion of radiotherapy. Seventeen (17) patients received pelvic radiation alone and 10 received both abdominal and pelvic radiation; cervical (n=13), Endometrial (n=5), synchronous endometrial and ovarian (n=1), and 3 ovarian cancers in the women. Eleven (11) patients had radiation followed gynaecological surgery (TAH with or without BSO/Omentectomy). Gastrointestinal symptoms, absorption of bile acids, vitamin B12, lactose and lipid levels were measured. Other endpoints analyses included gastric emptying, small-intestinal and whole-gut transit, stool weight and intestinal permeability.

Results were compared with those from 18 normal volunteers. All 27 patients completed at least 2 series of measurements and 18 completed all 5. Increased stool frequency during radiation treatment was associated with decreased bile acid absorption and vitamin B12 absorption, increased faecal fat excretion, increased presence of lactose malabsorption and faster whole gut transit. The investigators reported an improvement of most of the changes with time, however at 1 year after the completion of irradiation, the frequency of bowel actions was greater, bile acid absorption was less, and small-intestinal transit was more rapid when compared with those at baseline and normal subjects.

Symptoms of delayed radiation-induced bowel injury may be due to mucosal atrophy dysfunction, or intestinal dysmotility leading to (small bowel) bacterial overgrowth. The manifestations of each feature can vary between patients and thus investigating clinical signs and symptoms can be difficult and should be individualised. Table 1.1 below demonstrates the patho-physiological features with possible signs and symptoms associated with these.

Pathophysiological Feature	*Clinical Sign or Symptom*	*Diagnostic Evaluation*
Mucosal dysfunction	Lactose intolerance	Lactose absorption test
	Vitamin B12 deficiency	Schilling test
	Steatorrhea	Fecal fat excretion test
		mucosal permeability tests
Stricture or stagnant loop syndrome with bacterial overgrowth	Diarrhea	Diagnostic imaging
		Breath tests
		Endoscopic sampling
Intestinal dysmotility	Bloating	Intestinal manometry
	Constipation	Functional imaging
	Diarrhea	Intestinal transit tests
Abnormal bile acid recirculation	Cholerrheic diarrhea	Specific breath tests to assess bile acid absorption and deconjugation

Table 1.1. Patho-physiological features (and Diagnostic Evaluation) of Delayed Radiation Enteropathy (Taken from Hauer-Jensen et al, 2003).

1.8.1 Endoscopic diagnostic features of Radiation-Induced Bowel Injury

The role of endoscopy in evaluating patients who present with symptoms of radiation-induced bowel toxicity (RIBI) is limited. No systematic and objective scoring for mucosal damage and findings at endoscopy specific to RIBI exists to date. A recent study (Kim et al, 2011) described chronic rectal mucosal damage after pelvic radiotherapy (RT) for cervical cancer in 32 women. The median follow-up for the cohort was 35 months after EBRT and ICB. The Vienna Rectal Score (VRS) was used to describe the endoscopic findings and was compared to the European Organization for Research and Treatment of Cancer (EORTC)/Radiation Therapy Oncology Group (RTOG) morbidity score and the dosimetric parameters of radiotherapy.

The ratio of rectal dose calculated at the rectal point [RP] was compared to the prescribed dose, the biologically effective dose [BED] at the RP during radiotherapy planning. Rectal symptoms were noted in 28/32 patients (rectal bleeding, n=21; bowel habit, n=6; per-rectal mucus, n=1). Four (4/32) patients had no symptoms. Endoscopic findings included telangectasia (n=18), congested mucosa (n=20), ulceration (n=50 and a stricture (n=1). The RP ratio and BEDs were found to be significantly associated with the

EORTC/RTOG score and thus severity of symptoms. Other earlier studies also suggest telengiectasia and congested mucosa to be the most prevalent endoscopic findings in radiation enteropathy/proctopathy (Hasleton et al, 1985; den Hartog Jager et al, 1985).

Endoscopic follow-up studies also suggest that late (rectal) mucosal changes improve over time with a peak at 12 months after treatment (Hovdenak et al, 2000; O'Brien et al, 2004). A high incidence of rectal damage however, is usually found when endoscopy is done to evaluate symptoms of severe rectal bleeding (Figures 1.5 and 1.6).

1.9 Histopathological Diagnostic Features

Radiation enteropathy is characterised by diffuse collagen deposition and progressive occlusive vasculitis. The vasculitis and fibrosis progress over time, resulting in narrowing of the intestinal lumen with dilatation of the bowel proximal to the stricture. The affected segments of the intestine and serosa become thickened. Ulceration, necrosis, and occasional perforation of the intestinal wall may occur (Hasleton et al, 1985). The increased expression of TGF-β is particularly enhanced in areas with histopathological changes consistent with radiation injury: mucosal ulceration, mucosal and serosal thickening, inflammatory cell infiltrates, and vascular sclerosis. Pathological examination of bowel specimens from patients who underwent surgery for radiation enteropathy showed an increased immunoreactivity of TGF-β in areas with vascular sclerosis and fibrotic areas of serosa and muscularis propia compared with patients who underwent surgery for other reasons (Richter et al, 1996). Figure 1.4 shows cellular changes seen in early and late effects of radiation.

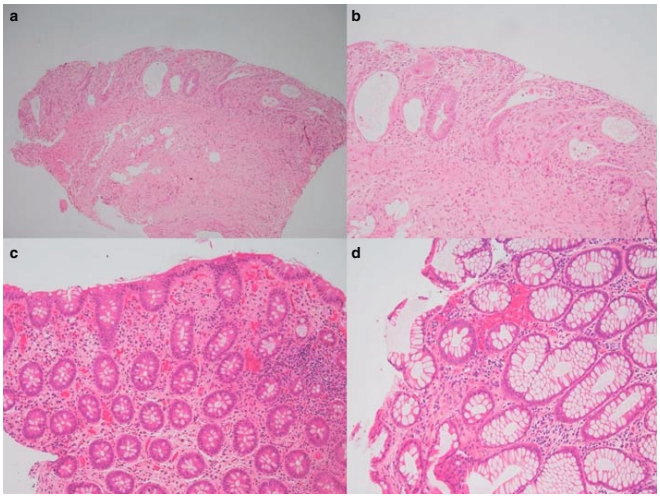


Figure 1.4. Cellular changes in Early and Late Effects of Radiation. Taken from Andreyev et al, 2011. (a) and b) Early effects. There is marked distortion of the mucosa. There are withered crypts lined by epithelial cells that have lost mucin and show toxic damage with abundant pale eiosinophilic cytoplasm. The crypts contain cellular debris. The nuclei contain prominent nucleoli. The lamina propria contains scattered plasma cells and eiosinophils. Endothelial cells are enlarged with large nuclei containing prominent nucleoli. (c) Late Effect. Large bowel mucosa shows fine fibrosis of lamina propria with dilated and small capillaries. Some crypts show mucin depletion. (d) Late Effect. There is crypt distortion and branching with fibrosis in the lamina propria that is slightly condensed around the crypts. An eosinophil is seen in the lamina propria.

1.10 Risk Factors for Radiation-Induced Bowel Injury

Inter-patient variability in normal tissue response to radiotherapy is well recognised. All tissue will express some level of response to radiotherapy at molecular, histological and clinical level, yet not all patients will develop injury significant enough to manifest as clinical symptoms. The incidence and severity of radiation-induced bowel injury is dependent on a number of patient and treatment factors (Table 1.1).

Patient factors such as low body mass index (BMI), presence of other co-morbidities; diabetes, hypertension, collagen disorders, and pre-existing inflammatory bowel disease (IBD) have been implicated with a higher risk of developing symptoms of RIBI (Chon

and Loeffler, 2002). A history of previous abdominal surgery predisposes to RIBI due to anatomical changes caused by bowel adhesions, leading to fixation of small bowel loops in the radiation field (Forbes, 2001; Letschert et al, 1990; Hauer-Jensen et al, 2014).

As discussed earlier, the total radiation dose and the volume of irradiated bowel (determined by the planning fields) remain the most important factors predisposing to the risk of RIBI. Evidence shows severe enteropathy and bowel injury is relatively rare with doses <50 Gy. The total dose at which 5% of patients are expected to experience enteropathy from RIBI at 5 years (TD5/5) is 50Gy for a third of the volume of the small bowel and 40Gy for the whole volume. The dose at which 50% of patients will develop enteropathy at 5 years (TD50/5) is 60Gy for a third of the volume and 55Gy for the whole volume. The tolerance for large bowel is slightly higher than for small bowel; TD5/5 of 55Gy for a third of the volume and 45Gy for the whole volume, with a TD50/5 of 65Gy for a third of the volume and 60Gy for the whole volume of the colon (Emami et al, 1991.

There is considerable evidence to show that extended field techniques and using anterior-posterior opposing fields can cause significantly more bowel toxicity than 3-4 field treatment (Letschert et al, 1990; Mak et al, 1994) and the more modern conformal CT planning techniques (Greven et al, 1991).

The addition of chemotherapy has also been shown to increase the rates of radiation - induced bowel injury however a systematic review of trials comparing concomitant chemo-radiation to radiation alone for the treatment of locally advanced cervical cancer concluded that, although concomitant chemo-radiation resulted in a two-fold increase in acute bowel toxicity, this was not found to be associated with late adverse effects (Kirwan et al, 2003).

From my review of the literature, I have compiled the evidence for risk factors as shown in table 1.2 below.

Treatment-Related Factors	References
Radiation Dose	*Emami B et al, 1991* *Roeske et al, 1997*
Volume/length of Irradiated bowel	*Emami et al, 1991*
Time-dose fractionation	*Dahlberg M et al, 1998* *Bujko K et al, 2006* *Hovdenak et al, 2000*
Concomitant Chemotherapy	*Ooi et al, 1999* *Letschert et al, 1990* *Kirwan et al, 2003*
Patient-related Factors	
BMI (LOW)	*Eifel PJ et al, 2002;* *Wedlake LJ et al 2010*
Previous Abdominal Surgery	*Forbes, 2001* *Letschert et al, 1990*
Previous Bowel Disorders (IBD*)	*Willett CJ et al, 2000*
Medical Co-morbidities – Diabetes, Vascular Disorders, Collagen disorders	*Herold D et al, 1999; Potish RA et al, 1983; Ross JG et al, 1993; Lin A et al, 2008*
Smoking	*Eifel PJ et al, 2002* *Chon and Loeffler, 2002*
Genetic Predisposition?	*West CM et al, 2011*

Table 1.2. Risk Factors for Radiation-induced Bowel Injury (Evidence from the Literature). IBD* - Inflammatory Bowel Disease (Ulcerative Colitis, Crohn's Disease)

1.11 Investigating symptoms of Radiation injury to the Bowel

In 2012 a practice guidance was designed and published by the Royal Marsden 'Pelvic Radiation Disease' Group (Andreyev et al, 2012) on behalf of the British Society of Gastroenterology, to facilitate clinical practice and improve management of patients with symptoms of RIBI in accordance with the goals set out by the National Cancer Survivorship Initiative (NCI Vision, 2010). Table 1.3 below summaries the suggested

algorithmic approach used by the Royal Marsden Hospital, UK for investigating symptoms of RIBI.

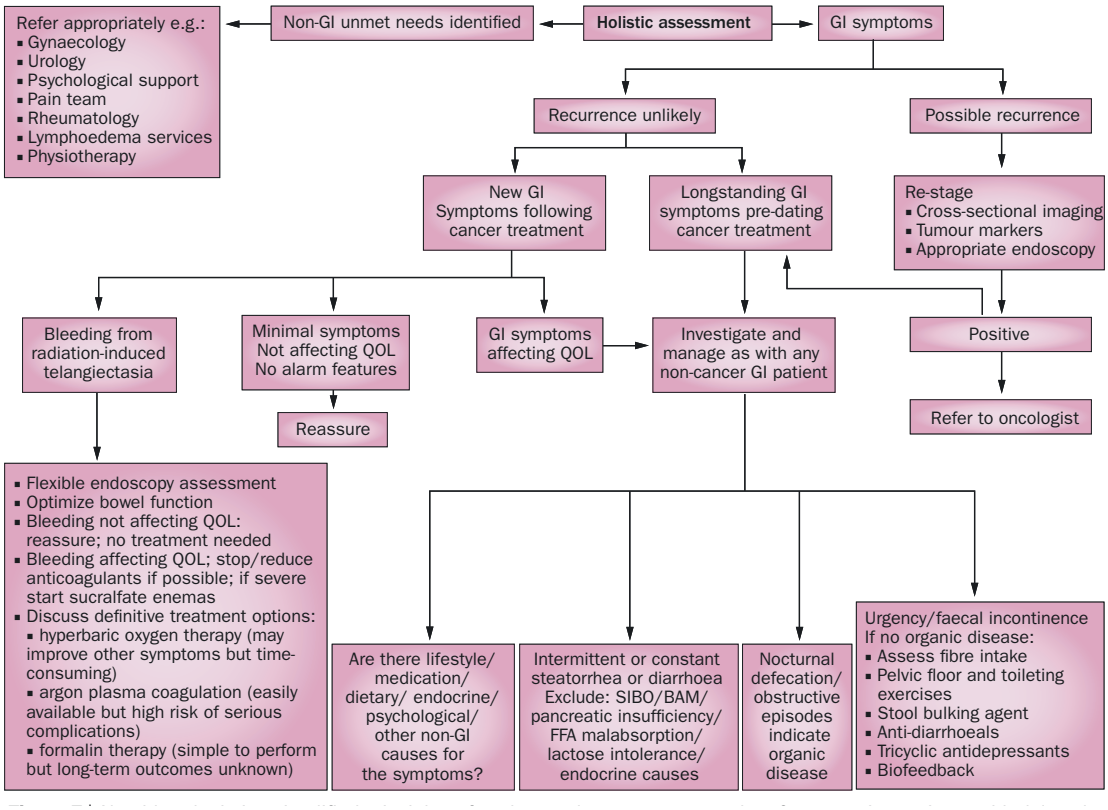


Table 1.3 Algorithm depicting simplified principles of work-up and common approaches for managing patients with delayed gastrointestinal symptoms after radiotherapy at the Royal Marsden London. Abbreviations; BAM, bile-acid malabsorption; FFA, free fatty acid; GI, Gastrointestinal; QOL, quality of life; SIBO, small intestinal bacterial overgrowth (Taken from Hauer-Jensen et al, 2014).

Current symptom questionnaires and scoring tools, such as LENT/SOMA (Late-Effects Normal Tissue taskforce – Subjective, Objective, Management, Analytical) and Radiation Therapy Oncology Group (RTOG) questionnaire have limited use in the clinical setting in assessing and evaluating patients symptoms; they are still mostly unable to direct diagnosis of underlying pathologies related to RIBI (Andreyev, 2007a,b).

Useful Initial investigations include:

- Vitamin B12 levels
- Thyroid function tests
- Coeliac Screen
- Selenium Homocholic acid taurine (SeCHAT) Test
- Glucose hydrogen/methane breath test
- Upper GI endoscopy + duodenal aspirate
- Flexible Sigmoidoscopy +/- Biopsy
- Computed Tomography (CT) Scan
- Magnetic Resonance Imaging (MRI) Abdomen and Pelvis

Initial laboratory tests are useful to exclude malabsorption and anaemia. As multiple sites of injury may contribute to symptoms, the choice of investigations are guided by symptoms; patients with rectal bleeding will have blood tests to exclude anaemia and will be referred to endoscopy to exclude new neoplasia. Investigation of symptoms of RIBI may show pathological changes in multiple areas of small and large bowel, which may not be related to presenting symptoms.

The role of imaging in investigating patients with gastrointestinal symptoms after radiotherapy has long been established and CT and MRI scans are part of initial investigations in patients presenting with symptoms of acute or sub-acute bowel obstruction to exclude radiation-induced strictures and fibrosis in both small and large bowel. An algorithmic approach to investigating symptoms should enable the structured evaluation of symptoms of RIBI.

1.12 Severe complications arising from Radiation-Injury to the Bowel

Patients can present with severe symptoms affecting quality of life after radiotherapy (Andreyev, 2007 & 2010) including:

- Transfusion-dependent rectal bleeding requiring intervention (Figures 1.5 and 1.6)
- Acute or sub-acute bowel obstruction requiring surgery and enteral feeding
- Intestinal Fistulas
- Bowel Perforation

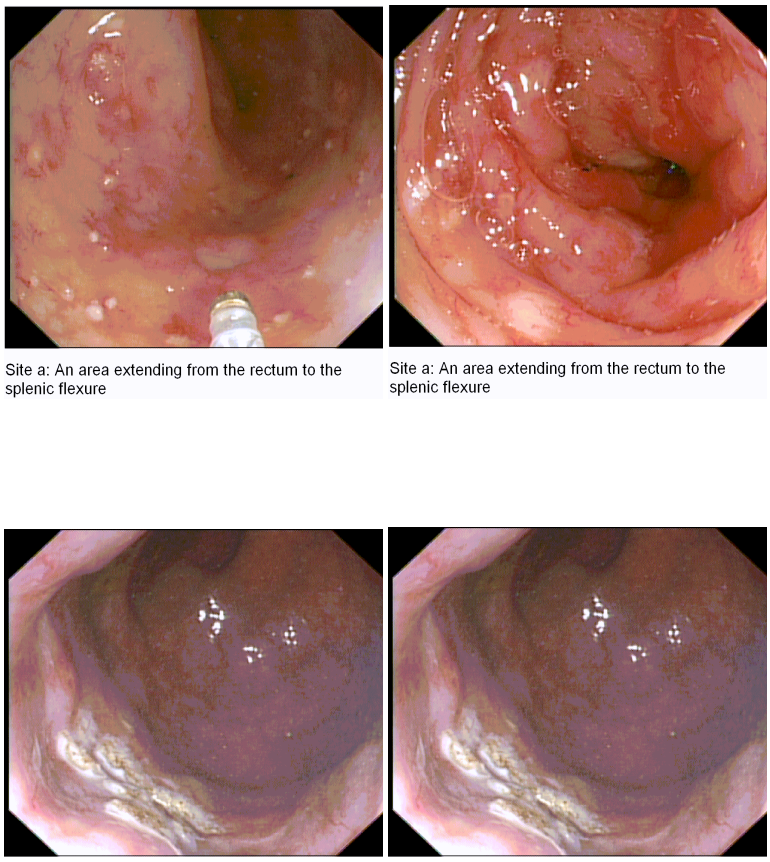


Figure 1.5 Transfusion-dependent Rectal Bleeding requiring endoscopic treatment. Case 1. This patient was part of our follow-up in our cohort study (chapter 2) and presented with recurrent significant rectal bleeding affecting her quality of life. She required Argon Beam Diathermy (APC) (2 separate treatments) to control symptoms

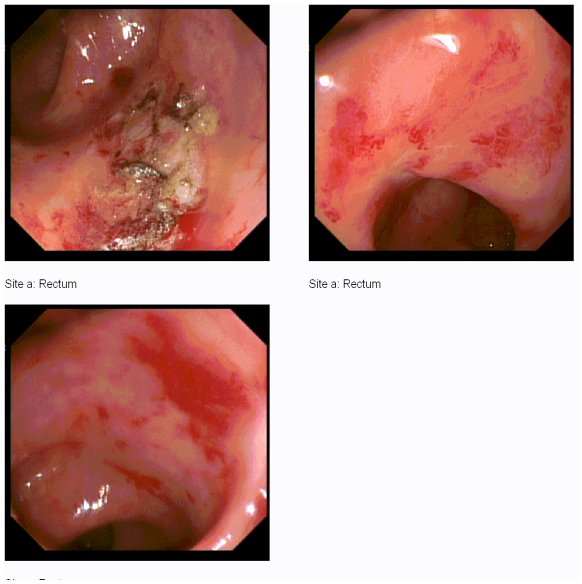


Figure 1.6. Case 2. Argon Beam Diathermy for moderate to severe radiation proctitis in a cervical cancer patient presenting with rectal bleeding**.**

1.13 Non-Surgical Interventions in the Management of Radiation-Induced Bowel Injury

Acute radiation enteropathy and the manifestations of late radiation injury to the bowel; 'pelvic radiation disease' – haemorrhages, fistulas, obstruction, abscesses, bowel perforations, strictures account for a large amount of morbidity in patients, affecting their quality of life, distrupting cancer survivorship with an increasing cost to healthcare.

Acute RIBI is mostly self-limiting and usually requires only conservative management. In most cases, only a small proportion of women require admission and inpatient management of dehydration with acute colitis or enteritis. Chronic RIBI (radiation enteropathy/colopathy/proctopathy) however is poorly predictable and a progressive disease manifesting in severe cases as a consequence of irreversible injury to the bowel. The management of chronic radiation injury to the bowel remains challenging, with surgery indicated only in severe cases with complications such as small/large bowel strictures with fibrosis, partial/complete obstruction, perforation and fistulas.

Despite the high prevalence of radiation-induced bowel injury, the evidence for effective treatments stil remain limited. Therapeutic options are available and wide-ranging and can be tailored to the clinical presentation and the underlying aetiology once identified. Denton et al, (2002) published a systematic review for non-surgical interventions for the management of late radiation proctitis. This study highlighted the lack of randomised controlled trials in proving the efficacy of most therapeutic options.

Diarrhoea in chronic enteropathy can be managed with ***anti-motility agents*** such as loperamide or codeine phosphate. ***Probiotics and Prebiotics*** alter the intestinal microbial environment and may prevent radiation enteropathy (Seal et al, 2007) and current trials are underway to support this. Bacterial overgrowth has been shown to respond to antibiotics (Danielsson et al, 1991; Meyers et al, 2001; Gasbarrini et al, 2007).

Cholestyramine is used to treat bile-acid malabsorption, which has been shown to account for 35-72% of symptoms in patients suffering from bowel symptoms following radiotherapy (Andreyev et al, 2005; Danielsson et al, 1991; Ludgate and Merrick, 1985). Rectal RIBI (radiation proctopathy) with bleeding can be managed with ***sulcrafate enemas*** (Kochhar et al, 1991; Sanguinetti et al, 2003). Rectal bleeding can be controlled with ***Argon beam therapy*** (endoscopic) or ***hyperbaric oxygen*** (Hauer-Jensen et al, 2014; Clarke et al, 2008).

The recent ORBIT trial (Andreyev et al, 2013) showed that patients' symptoms can be helped significantly if a systematic algorithmic approach is used to elicit symptoms. This trial also showed that a nurse trained to manage symptoms can be as effective as a senior gastroenterologist, especially with limited resources and long waiting times for referrals to gastroenterologists by oncologists.

1.14 Surgical Management of Radiation-Induced Bowel Injury

The incidence of severe RIBI requiring surgical intervention remains significant and has been stated as being up to one-third of all patients receiving curatve doses of pelvic radiation (Andreyev, 2007). In one cohort study, (Lefevre et al, 2011) approximately one-third of patients with chronic radiation enteropathy required surgery, which was associated with a high morbidity rate and a high risk of re-operation. The aim of this study was to report outcome after surgery for radiation enteropathy. The cohort consisted of 107 patients; (94 women; 87.8%). The main indication for surgery was symptomatic stricture (76.6%). Forty-nine (49) ileo-caecal resections (45.8%) were performed. Overall and surgical morbidity rates were 74.8% (80 patients) and 28% (20) respectively. Fourteen (14) patients (13.1%) underwent re-operation for complications after the initial surgery. Re-operation rates at 1 and 3 years of follow-up were 37 and 54% respectively. Risk factors for reoperation or recurrent enteropathy were: emergency surgery (OR 2.72, 95% CI 1.57-4.86), anastomotic leakage (OR 2.53, 95% CI 1.54-4.42) and male sex (OR 3.57 95% CI 1.87-7.29). The only protective factor for re-operation was ileo-caecal resection (compared to intestinal bypass surgery or adhesiolysis) during the first surgical operation (OR 4.48 95% CI 2.52-8.31).

Regimbeau et al (2001) reported 109 patients (62% with gynaecological cancer) with chronic radiation enteropathy (mean radiation dose 55Gy +/- 17Gy (range 10 to 105Gy) between 1984 and 1994 were operated on with a mean follow-up of 40 months (range 1 - 293). Five (5) patients died in the post-operative period. Operative mortality was significantly higher in the resection group (5% vs 0%). Thirty-three (33; 30%) of patients experienced post-operative complications including anastomotic leak in 11. At the end of follow-up, parenteral nutrition (TPN) remain mandatory for 18 (32%) patients from the resection group versus 14 (38%) in the conservative group. The difference was not significant. Twenty-one patients (21; 19%) at follow-up were diagnosed with short bowel syndrome (<1m) associated with diarrhoea.

Gavazzi et al, (2006) compared the long term outcome of patients with radiation-induced intestinal obstruction treated either surgically or with intestinal rest and home parenteral nutrition (HTPN), 17 patients underwent surgery and 13 were supported by HTPN. Fifty percent (50%) of these patients had been treated for gynaecological cancers. Long term nutritional autonomy and survival seemed to be better in patients initially treated with intestinal rest and HTPN; 46% required surgery after a mean period of 12.7 months of nutritional support because of persistence of symptoms supported by radiological evidence indicating surgical intervention.

1.15 Preventing and Reducing Normal tissue effects from Pelvic Radiation

Interventions to reduce the incidence of RIBI and protect healthy tissue from radiation effects, whilst maintaining optimal tumour control remain largely unavailable. Previous and ongoing studies have explored various strategies. As radiation-specific injury is initiated by reactive oxygen species, antioxidant, free radical scavengers and various cyto-protectors have been subjects of study for decades. Amifostine, a potent scavenger of free radicals has been investigated for the prevention of RIBI (Ben-Josef et al, 2002). Superoxide dismutase has also been explored as a potential for radio-protection (Salvemini et al, 2002).

Other interventions to prevent toxicity involve exploring the modulators of the patho-physiological or cellular responses to radiation by seeking agents that can increase radiation tolerance, ameliorate secondary normal tissue injury, or enhance repair capacity (Hauer-Jensen et al, 2014).

1.16 Advances in Radiobiology: Optimising Planning and Delivery techniques

Intensity Modulated Radiotherapy (IMRT)

For most pelvic tumours radiotherapy remains a mainstay of treatment, and in gynaecological cancers especially for locally advanced cervical cancers. Newer techniques such as IMRT, which allows a higher degree of conformity to tumour, provides the opportunity, to further reduce radiation related toxicity whilst also potentially improving local control by increasing tumour dose. By varying the intensity of the radiation beam, IMRT allows more shaping of radiation fields even around concavities. The dose distribution of IMRT fits more precisely to the target volume, producing a concave shape at the posterior aspect of the PTV, reducing the dose to the rectum, and also anteriorly, curving around the lateral lymph node target volume while sparing more of the central bladder and bowel (Powell, 2010).

Mundt et al, (2001) have sown a reduction in Grade 2 gastrointestinal toxicity from 91% to 60% in a cohort of 40 patients (70% post-operative, 40% primary radiotherapy). Late toxicity was reduced from 50% to 11%. Portelance et al (2001) showed in a dosimetric study that IMRT reduces small bowel, rectum and bladder doses in patients with cervical cancer receiving pelvic and para-aortic irradiation. CT scans studies of 10 patients with cervical cancer were used as anatomic references for planning. Upon completion of target and critical structure delineation, the imaging and contour data were transferred to both an IMRT planning system (Corvus, Nomos) and a three-dimensional planning system (Focus, CMS) on which IMRT as well as conventional planning with two- and four-field techniques were derived.

Treatment planning was done on these two systems with uniform prescription, 45Gy in 25 fractions to the uterus, cervix and the pelvic and para-aortic lymph nodes. Normalisation was done to all IMRT plans to obtain a full coverage of the cervix with the 95% isodose curve. Dose-volume histograms were obtained for all the plans. The volume of small bowel receiving the prescribed dose (45Gy) with IMRT technique were: four fields, 11.01 +/- 5.67%; seven fields, 15.05 +/- 6.76% and nine fields, 13.56 +/-

5.30%. These were all significantly better than with two-field (35.58 +/- 13.84%) and four-field (34.24 +/- 17.82%) conventional techniques ($p<0.05$). The fraction of rectal volume receiving a dose greater than described was as follows; four fields, 8.55 +/- 4.64%; seven fields, 6.37 +/-5.19%, nine fields, 3.34 +/- 3.0%; in contrast to 84.01 +/- 18.37% with two–field and 46.37 +/- 24.97% with four-field conventional technique ($p<0.001$). This study demonstrated that with similar target coverage, normal tissue-sparing is superior with IMRT compared to convention radiotherapy in cervical cancer treatment.

1.17 Improving uncomplicated cancer cure rates and the Cancer Survivorship Initiative

There is a need to identify tissue specific biomarkers of normal tissue injury and identify those patients who might be at risk of severe injury to the bowel. Patients with cervical cancer especially are usually young and fit with symptoms that significantly affect their quality of life.

Prospective trials and research into the better understanding of the patho-physiology of RIBI continue to be difficult given the complex relationship between symptom presentations these patients and the nature of the toxicity and RIBI.

In this thesis I will attempt to answer the following questions and explore the following:

1. True incidence and presentation of RIBI in a London Cancer Centre.
2. How scoring and reporting of symptoms might be improved.
3. Can Cell-cycle markers of radiotherapy be used as a marker of (chemo)-radiosensitivity?
4. Whether cell-cycle markers in colonic crypt cells can shed more light on the proliferative status after exposure to radiation and if this linked to severity of RIBI, grade of histopathological features and other endpoints.

Chapter Two A Retrospective-Prospective Cohort Study

Chapter Two A Retrospective Cohort Study

Radiation-Induced Bowel Injury: The Impact of Radiotherapy after treatment for Gynaecological Cancers

2.1 Introduction

The true prevalence of late bowel toxicity in the UK remains unknown due to the paucity of prospective studies in patients who following completion of radiotherapy. Many patients are discharged from oncology centres follow-up after 2-5 years, and some are investigated and treated for bowel symptoms and complications in other units/hospitals. The reporting of late toxicity remains poor; patients rarely report mild chronic symptoms of loose stools and diarrhoea, or those symptoms not considered to be related to treatment, or 'embarrassing' symptoms like flatulence and faecal incontinence/leaking. Although the majority of women who receive pelvic radiation will present with symptoms of acute radiation-induced bowel injury ('Radiation Enteritis/Proctitis/Proctopathy'), which may be present during and up to 3 months after treatment, some of these women report symptoms settle and a return to normal function (Andreyev 2007a). It remains unclear why only a proportion of women (5-50%, depending on study) (DeCosse et al, 1969; Galland and Spencer, 1985; Theis VS et al, 2010) never settle or indeed some go on to represent months or years after pelvic radiotherapy with symptoms of radiation-induced bowel injury or 'Pelvic Radiation Disease'.

2.2 Materials and Methods

2.2.1 Study Cohort

The records of 541 patients diagnosed between February 2003 and June 2010 within the North London Cancer Gynaecological Cancer Network who required radiation as part of their treatment were reviewed; 219 patients with histologically confirmed Cervical Cancer- International Federation of Gynaecology and Obstetrics (FIGO) stages IB2-IVA and 322 women with Endometrial Cancer FIGO stages IB-IV treated with radiotherapy with or without surgery and/or chemotherapy. Inclusion criteria included patients with both cancer types who had received a 'treatment' dose of radiation according to standard protocols at our centre who then reported symptoms of bowel toxicity requiring investigation or referral to a gastroenterologist. Patients who presented with symptoms after 3 months from completion of radiotherapy were included in the study as well as those who reported 'chronic' symptoms that had not subsided or improved since the end of treatment. Exclusion criteria included patients who received a 'palliative' dose (20-30Gy) of radiation for recurrence or advanced disease, and patients who reported mild symptoms immediately after completion of radiotherapy that had settled by the next subsequent follow-up.

2.2.2 Data Collection

Oncology and radiotherapy databases were searched to identify all patients who had received radiotherapy with curative intent. The gastroenterology database was also searched to include all patients referred from oncology for investigations of bowel symptoms following radiotherapy. Clinical data on cancer demographics, treatment received, outcome and follow-up was obtained from hospital records. Original pathology reports were reviewed for histological type, FIGO stage, and tumour grade. I extracted data on: date of diagnosis, past medical and smoking history, date and type of primary

surgery, stage of disease, type and dose of radiotherapy, and date of completion of radiotherapy, chemotherapy received, disease recurrence or progression; date of presentation with bowel symptoms, nature of symptoms and degree to which symptoms affected quality of life (QoL), investigations and treatment of chronic radiation-related bowel symptoms, as well as status at last oncology and gastroenterology follow-up were all also obtained from individual patient records, clinic letters, and review of imaging reports and other investigation results.

Patients were followed up in the multidisciplinary oncology clinics every 3 months for 2 years, then 6 monthly for 2 (early stage endometrial), and annually to 10 years (locally/(advanced) stage cervical) depending on cancer type and stage. Clinical examination and routine screening bowel and bladder toxicity screen were undertaken and documented. Patients were referred to gastroenterologists if bowel symptoms were moderate and deemed to be affecting the patients' quality of life or if they had symptoms that required investigations (for example rectal bleeding). Computerized Tomography (CT) or Magnetic Resonance Imaging (MRI) was undertaken if clinically indicated. Patients referred from neighboring cancer units initially were followed up at the centre for 2 years and were then referred back to the regional units if there were found to be free of disease after 2 or more years.

Treatment Received

Cervical Cancer;

Chemotherapy

Majority of patients FIGO (IB-IVA) received concomitant chemo-radiation with weekly Cisplatin 40mg/m2.

Radiotherapy

Radiotherapy was given according to our defined cancer centre's protocol - concurrently with weekly Cisplatin; almost all patients received primary radiotherapy stages (IB - IV): external beam radiation (EBRT) to the pelvis (50.4Gy in 28 fractions over 5.5 weeks using 8-15mV photons) and intracavity brachytherapy (ICB) using an intrauterine ovoid system (15Gy in 2 fractions/HDR/point A). Extended fields were used to treat para-aortic lymph nodes (PALN) using an AP/PA field to a dose of 45Gy in 25 fractions over 5 weeks. Where parametrial invasion or pelvic side wall extension was evident, a further 5.04Gy in 3 fractions was delivered to the pelvic side wall (n=27, 38%).

Surgery

Patients were selected based on risk determined by clinical staging for laparoscopic para-aortic node dissection. Primary surgery was not indicated for locally advanced stage disease in the majority of patients.

<u>Endometrial Cancer;</u>

Surgery

Primary treatment for all patients involved a total hysterectomy with bilateral salpingo-oophorectomy (TAH/BSO) with peritoneal washings.

Chemotherapy

When adjuvant chemotherapy was given, patients received 6 cycles of carboplatin AUC 5 and paclitaxel 175mg/min2 every 3 weeks. This was usually within 4 weeks after primary surgery.

Radiotherapy

Radiotherapy to the pelvis was delivered using external beam radiotherapy at a dose of 45Gy in 25 fractions over 5 weeks with an additional 12Gy in 2 fractions to the vaginal vault. Radiation treatment started immediately after recovery from surgery or chemotherapy if received, and was usually within 2-4 weeks of completion of chemotherapy.

Radiotherapy Planning

Until 2006, radiotherapy was planned using orthogonal films, and delivered using four orthogonal fields. From 2007, radiotherapy was delivered using 3-D conformal radiotherapy, planned using a dedicated CT planning scan, and with IV contrast unless contra-indicated. The planning target volume (PTV) was defined as the clinical target volume (CTV) (obtained form pre-treatment MRI and EUA) with a 10mm expansion. The external beam radiation to the pelvis was delivered using a four-field (AP/PA and two lateral fields) arrangement.

The conventional pelvic field extended from the top of L5 to the bottom of the obturator foramen or 2cm below the lowest level of disease and laterally 1.5cm beyond the bony pelvis. The lateral fields extended from the anterior border of the symphysis pubis to the S2/3 interspace posteriorly.

2.2.3 Data Analysis

Comparison between patient and treatment groups was done using Chi-squared analysis for discrete variables. Student's t-test was used to compare means. Risk factors/predictors for severe late bowel injury were assessed using univariate and multivariate ordinal logistic regression. Survival data were analysed using log-rank test

and Kaplan-Meier method was used to show time course and chronicity of late bowel symptoms related to survival.

Factor analysis was used to assess associations between the multiple presenting symptoms and correlate this with our data set outcomes. Survival was defined from date of completion of cancer therapy to date of last follow-up or death. Status of bowel injury symptoms/disease was defined as the interval from first documented presentation with bowel symptoms to date last seen by gastroenterologists or late toxicity status recorded by oncologists. A p value <0.05 was considered significant. Statistical analyses were performed with SPSS version 19.0.

2.2.4 Factor Analysis

As majority of patients presented with multiple signs and symptoms, we sought to find correlation between symptoms in different subsets or 'clusters'. Factor analysis tells us what variables in a set of data 'group' or 'go together' (Schmitt, 2011). This method of analysis explores the underlying variance structure of a set of correlation coefficients. If the analysis is designed to account for only the variance in the correlation coefficients and ignore the error variance (i.e., the variance not accounted for by the correlation coefficients), it is called a 'Factor Analysis' (Stapleton, 1997). If the analysis is designed to account for all the variance including that found in the correlation coefficients and error variance, it is called a ***Principal Components Analysis***. The idea behind the choice of this method was to take into account all presenting symptoms within each group of symptoms patients reported, as well as the pattern of variable 'groupings' in the entire cohort. In both types of analyses, the factors that underlie the correlations involved in all 'groupings' are calculated.

Factor analyses study the construct validity of a model. In convergent validity similar tests/items load together, whilst in divergent validity unrelated tests load separately. Both are used to reduce errors in the model. The ***factor loadings*** calculated is the correlation

of each of the items/variables within each factor or 'cluster'. The communalities represent the total proportion of variance that the analysis accounts for in each item.

Oblimin rotation reduces the cross-loadings between the factors. The Kayser-Myer-Olkin (KMO) statistic and Bartlett's test of Sphericity were also calculated to determine the factorability of the data.

Eigen Values

Determining the number of factors (i.e. clusters) to extract is traditionally based on Eigen values greater than 1 (Gaskin and Happell, 2014) and visual inspection of the scree plot. Eigen values fit into the overall picture as they decide the number of 'factors' to use in such an analysis. They represent the amount of 'information' captured by each factor.

A ***Scree Plot*** shows Eigen values on y-axis and the number of possible factors on the x-axis. It always displays a downward curve. The point where the slope of the curve levels, the 'elbow', indicates the number of factors that should be generated by the analysis.

2.3 Results

2.3.1 Patients

The clinico-pathologic variables and characteristics of patients are summarised in Table 2.1. Documented evidence was available for 152 women treated for gynaecological cancers (cervix; n=77, endometrial; n=75) at our oncology department who subsequently reported symptoms suggestive of radiation-induced bowel injury. Almost half of patients with cervical cancer were treated for stage IIB disease (35/77; 45.5%) with moderately differentiated tumours; grade 2 (47/77, 62%). Majority were squamous cell carcinomas –

57/77; 74%). Thirty-two (41.6%) women with cervical cancer were smokers. Twelve (16%) patients had a previous diagnosis of hypertension and 2 patients with type II diabetes, whilst 4 patients had been previously diagnosed with irritable bowel syndrome (IBS).

Seventy-five (75) patients treated for primary endometrial tumours were found to have reported new gastrointestinal symptoms after completion of radiotherapy, presumed to be radiation-induced. Over 50% of women had stage I/II disease: IC (26/75) and IIB (21/75) endometrial cancers; grade 2 tumours (46/75, 61%) and adenocarcinomas (61%). Only 5 of the women reported as smokers. Four (4) patients also had IBS and 5 had a previous history of diverticular disease (none in the cervical cancer group). One of these women had had a previous bowel perforation related to diverticular disease (and one of only 5 women in the endometrial cancer group who subsequently required surgery for resection of radiation-induced small bowel stricture). Median age was 52 years in the cervical cancer group and 63 years in the endometrial cancer group.

Primary Cancer	**Endometrial**	**Cervical**
Total no of patients reviewed	322	219
Patients presenting with significant symptoms of chronic treatment-related bowel injury (% of total)	75 **(23%)**	77 (**35%)**
	Frequency (%)	
Median age (range)	63 (40 -80)	52 (27-81)
Stage		
I	32	11
II	23	51
III	18	10
IV	0	4
Recurrence	3	1
Grade		
1	3	3
2	40	40
3	25	25
Unknown	5	10
Histology		
Squamous Cell	0	57
Adenocarcinoma	61	17
Adenosquamous	0	1
Uterine Serous Papillary	3	0
Mixed*	6	0
Small Cell	0	1
Clear Cell	1	1
MMMT	7	0
Smoking**		
Yes	5	32
No	63	36
Ex (>5yrs)	5	6
Past Medical History		
Bowel - IBS	4	4
Bowel - Diverticular disease	5	0
Hypertension	2	12
Diabetes (type II)	4	2
Interval to Presentation (months)		
Median (range)	10 (1 - 61)	8 (1 – 106)

Table 2.1. Patient Characteristics and Cancer Demographics.

Treatment – Cervical Cancer

Chemotherapy: Over 96% patients received 5 cycles of Cisplatin or more. Sixteen (21%) patients also received neo-adjuvant chemotherapy with 6 weeks of weekly Carboplatin and Paclitaxel within a clinical trial (McCormack et al, 2013). Two patients received Carboplatin and Etoposide for small cell carcinomas. Two patients did not receive any chemotherapy due to elderly age and significant co-morbidities.

Radiotherapy: 75/77 patients received external beam radiotherapy (50Gy) and intra-cavity brachytherapy as described in the methods. One patient, (stage IVA) underwent pelvic radiotherapy following hysterectomy: external beam radiation to the pelvis (45Gy in 25 fractions over 5 weeks/10MV photons) and vault brachytherapy (13Gy in 2 fractions/HDR/0.5cm from surface of applicator), whilst another, (stage IVB) had consolidative pelvic radiotherapy 40.0Gy in 5 fractions over 3 weeks after 6 cycles of Cisplatin and Topotecan for advanced disease.

Surgery: Fifteen (15) women underwent laparoscopic para-aortic node dissection as part of staging. There was only one major peri-operative complication; bowel injury due to a peri-operative complication with subsequent laparotomy with resection of perforated sigmoid and hartmann's colostomy, this patient went on to suffer severe late toxicity requiring a right hemi-colectomy. Only 3 women had surgery (total abdominal hysterectomy and bilateral salingo-oophorectomy - TAH/BSO) prior to (chemo)-radiation; 2 of these were incidental diagnoses on pathology.

Treatment – Endometrial Cancer

Chemotherapy: 27/75 (36%) received 5/6 cycles of Carboplatin and Paclitaxel chemotherapy prior to radiation treatment.

Radiotherapy: All patients received external beam radiotherapy, as described in methods.

Surgery: Forty-three (43) patients underwent a total abdominal hysterectomy and bilateral salpingo-oophorectomy (TAH/BSO) while 32 patients had a laparoscopic procedure (TLH/BSO). One patient was an incidental pathological diagnosis on a vaginal hysterectomy specimen (done for prolapse). Surgical staging also involved pelvic and/ or para-aortic lymphadenectomy in 14 patients. Five (5) had an omentectomy as part of staging to exclude metastatic disease.

Presenting Signs and Symptoms of Bowel Injury after Treatment

We identified 14 common 'new' bowel symptoms/signs reported and recorded by oncologists at follow-up; patients usually had multiple symptoms (Table 2.2). Patients who reported symptoms affecting quality of life were referred to gastroenterologists; 90% (69/77) cervical cancer patients, and 83% (62/75) of the endometrial cancer patients. Median time to presentation with bowel symptoms after completion of radiotherapy (or chemo-radiation) was 8 months (1 month – 9 years) in the cervical cancer group and 10 months (1 month – 5 years) in women treated for endometrial cancer.

Figure 1.1 shows the interval time to presentation (in oncology outpatient clinic or emergency department with acute bowel obstruction) from completion of radiotherapy. There was no statistical difference in interval from completion of radiotherapy to presentation between cancer types.

	Patient Group				
	Cervical Cancer (n=77)		**Endometrial Cancer (n=73)**		ALL (n=152)
	n	% of total	n	% of total	n (%)
Abdominal Pain	38	49.4	34	45.3	72 (47.4)
Bloating	22	28.6	23	30.7	45 (29.6)
Nausea	12	15.6	7	9.3	19 (12.5)
Vomiting	12	15.6	5	6.7	17 (11.2)
SABO[a]	19	24.7	9	12.0	28 (18.4)
ABO[b]	6	7.8	2	2.7	8 (5.3)
Diarrhoea	47	61.0	36	48.0	83 (54.6)
BO<4/day	51	66.2	49	65.3	100 (65.8)
BO>4/day	26	33.8	20	26.7	46 (30.3)
Urgency	62	80.5	62	82.7	124 (81.6)
Faecal Incontinence/leaking	26	33.8	16	21.3	42 (27.6)
Flatulence	12	15.6	12	16.0	24 (15.8)
PR bleed	28	26.4	28	37.3	56 (36.8)
PR mucus	7	9.1	6	8.0	13 (8.6)
SAQOL	32	41.6	28	37.3	60 (39.5)

Table 2.2. Reported signs and symptoms of radiation-induced bowel injury. Patients presented with multiple symptoms, with defeacation urgency being the most common presenting symptom. BO= number of times 'bowels opened' (frequency); SAQOL = patients reporting symptoms 'affecting their quality of life'; SABO[a] - symptoms of intermittent sub-acute bowel obstruction; ABO[b]- symptoms of acute bowel obstruction presenting as an emergency.

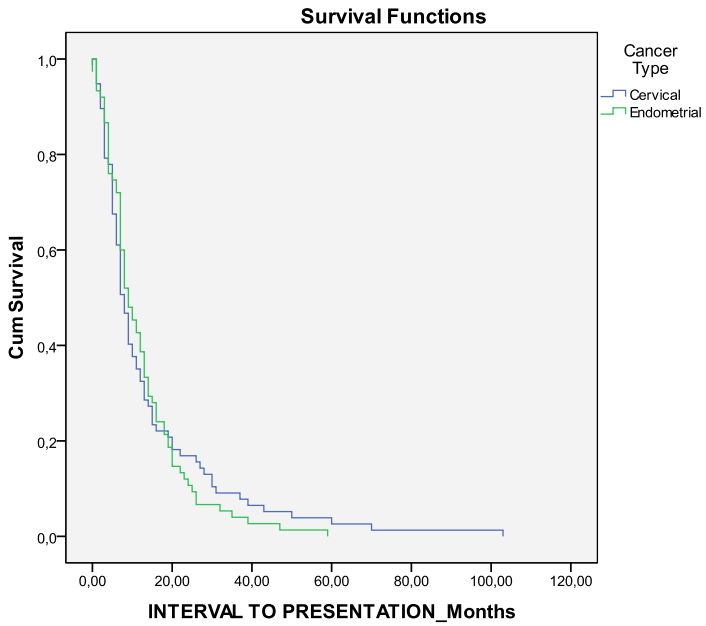


Figure 2.1. Interval to Presentation with symptoms of radiation-induced bowel injury.

Defecation urgency was the most common reported (and documented) symptom in 124/152 (81.6%) of women. Diarrhoea was reported in 54.6% (83/152) of women; 30.3% of women reported increased frequency with bowels frequency over 4 times a day (range 5 - 10). Twenty-eight women (18.4% of entire cohort) presented with, or reported symptoms and signs of intermittent sub-acute bowel obstruction (SABO) at oncology follow-up. Six (6) women in cervical cancer group, and 2 in endometrial cancer group presented to emergency department with symptoms and signs of acute bowel obstruction (ABO). Forty-two (27.6%) women reported faecal incontinence or admitted to leaking on questioning at gastroenterology assessment, whilst 36.8 % (n=56) of women in the entire group reported rectal bleeding after radiotherapy.

2.3.2 Factor Analysis – Three Factor Solution

Principal components analysis (PCA) was first used to assess the suitability of data for factor analysis. Inspection of the correlation matrix revealed the presence of many

coefficients 0.300 and above. The Kaiser-Meyer-Olkin value was 0.659, exceeding the recommended value of 0.600 (Kaiser 1974), and Bartlett's Test of Sphericity reached statistical significance (Bartlett 1954), supporting the factorability of the correlation matrix. PCA (Armstrong and Soelberg, 1968) revealed the presence of three components, with Eigen values exceeding 1, explaining 24.1%, 15.1%, and 13.3%. An inspection of the scree plot revealed a clear break after the third component. Using Cattell's (1966) scree test, we split the 'symptom clusters' to three components. This was further supported by the results of parallel analysis, which showed only three components, with Eigen values exceeding the corresponding criterion values for a randomly generated data matrix of the same size (14 variables × 152 respondents). The three-component solution explained a total of 52.5% of the variance. To aid in the interpretation of these, three components Oblimin Rotation was performed. Factor loadings are shown in Table 2.3. The 3-factor solution demonstrated minimal cross loadings. All 14 items (symptoms and signs) were retained and all except for 'PR mucus" (n=13 women recorded this variable), had loadings >0.3.

Symptoms/Signs	Pattern Matrix			Structure Matrix			Communalities
	Component			Component			
	1	2	3	1	2	3	
Nausea	**0.889**			**0.893**			0.799
Vomitting	**0.869**			**0.874**			0.767
SABO	**0.817**			**0.825**			0.701
ABO	**0.547**			**0.548**			0.352
BO>4d		**0.915**			**0.895**		0.794
BO<4d		**-0.847**	0.333		**-0.808**		0.849
Diarrhoea		**0.545**			**0.562**		0.34
FI		**0.507**			**0.543**	0.308	0.401
Bloating	0.365		**0.695**	0.354		**0.689**	0.608
Flat			**0.589**			**0.591**	0.373
Abdo pain	0.517		**0.546**	0.508		**0.537**	0.555
Urgency		0.367	**0.46**	-0.341	0.427	**0.486**	0.479
PRB	-0.303		**-0.339**			**-0.338**	0.205
PR mucus			**0.339**			**0.336**	0.124

Table 2.3. Factor Analysis; Component loadings for presenting symptoms of bowel injury.

ABO: Acute bowel obstruction; FI: Faecal Incontinence; PRB: Per-rectal bleeding; PR mucus: per-rectal mucus; SABO: Sub-acute bowel obstruction. Oblimin rotation generates both a pattern matrix and a structure matrix. The structure matrix is simply the factor loading matrix as in orthogonal rotation, representing the variance in a measured variable explained by a factor on both a unique and common contributions basis. The pattern matrix, in contrast, contains coefficients that represent unique contributions of each variable/item within the structure (similar to correlation coefficient). For Oblimin

rotation, the researcher looks at both the structure and pattern coefficients when attributing an item to a factor. A factor loading of over 0.3 in absolute value is considered to indicate which item/variable belongs to a factor; in any event factor loadings must be interpreted in light of theory, not on arbitrary cut-off levels. Loadings which indicate which variables where assigned to each component are highlighted in bold. The variance – the sum of the squared factor loadings for each item/variable (row). The communality measures the percent of variance in a given item/variable explained by all the factors jointly and is used as the reliability of the indicator.

Component 1 suggest '*Obstructive' Symptoms*, because it maintains high loading in symptoms suggestive of (radiotherapy-induced) bowel stricture/obstruction (nausea, abdominal pain, vomiting, signs and symptoms of intermittent sub-acute bowel obstruction (SABO), and acute bowel obstruction (ABO)). Component 2 – '*Enteropathy' Symptoms* (diarrhoea, loose stools and increased bowel frequency, and faecal incontinence) suggests symptoms of small bowel dysfunction, while component 3 is more pathgnomonic, (though not definitive) of '*Colitis-Proctitis/Proctopathy' Symptoms* (bloating, flatulence, abdominal pain, faecal urgency, per-rectal bleeding (PRB), and per-rectal mucus (PR mucus)). Factor scores were computed and used in subsequent analyses. The purpose of this type of analysis and 'clustering' presenting symptoms was to try to identify, within our cohort of women, associations and possible predictors of, disease course and severity, site of bowel injury and underlying patho-physiological processes.

2.3.3 Predictors of Severity and Chronicity of Bowel Problems

Patients were followed up until symptoms resolved or until their last oncology/gastroenterology follow-up prior to the end of data collection in December 2012. Information on status of bowel symptoms (and disease/general health) was obtained from documentation at these consultations. Table 2.4 shows a summary of documented status at last follow up.

	Cervical	Endometrial	ALL	
	n	n	n	%
Alive and well, Bowel symptoms resolved	21	21	42	**27.6**
Alive with Disease Progression	3	2	5	**3.3**
Alive, ongoing Bowel symptoms				
➢ *Mild, managed with Imodium/Diet*	25	38	63	**41.4**
➢ *Moderate, awaiting further investigations*	4	1	5	**3.3**
➢ *Severe, symptoms affecting QoL*	19	2	21	**13.8**
Deceased			16	**10.6**
➢ *Dead, recurrent disease*	3	9	12	
➢ *Dead, secondary to enteritis complications*	1	0	1	
➢ *Dead, other causes*	1	2	3	

Table 2.4. Patient Status at last follow-up

At the time of analysis, 42/152 (27.6%) of women were alive, disease-free with resolution of bowel symptoms. Five patients (3.3%) were alive with disease progression,

without clear documentation of follow-up of late bowel toxicity symptoms. 63/152 (41.1%) of all patients were disease-free with ongoing mild symptoms managed by oncologists and/or discharged from gastroenterology follow-up. Majority of these patients had mild urgency, and frequency with loose stools, with bowel motions on average twice a day. Most patients reported stable symptoms not affecting quality of life and some required anti-motility drugs (Imodium) intermittently with dietary manipulation to control symptoms. Five women (3.3%) had ongoing moderate symptoms, with significant diarrhoea, frequency and urgency and were undergoing further investigations.

Twenty-one patients (21) – 13.8% of patients still had ongoing severe symptoms affecting their quality of life, some even after surgical intervention. Only 2 of these patients had been treated for endometrial cancer whilst 19 had received concurrent chemo-radiation for a cervical primary cancer. Median follow-up time (from presentation with symptoms of radiation-induced bowel injury) was 89 months for the patients who had resolution of their symptoms at last follow-up; 37.75 months for patients with mild manageable symptoms; 50.5 months for the 5 patients with ongoing moderate symptoms (mostly urgency and diarrhoea); and 34.5 months for patients with ongoing severe symptoms of radiation-induced bowel injury at last follow-up. There was no statistical significant difference when comparing follow-up time and status (resolution or degree of severity) at last follow-up.

Figure 2.2 shows the follow-up time from presentation with bowel symptoms. We found a significant difference between cancer types (log rank $\chi^2 = 8.065$, $p=0.005$) suggesting women with endometrial cancer were more likely to have less severe, but more chronic symptoms.

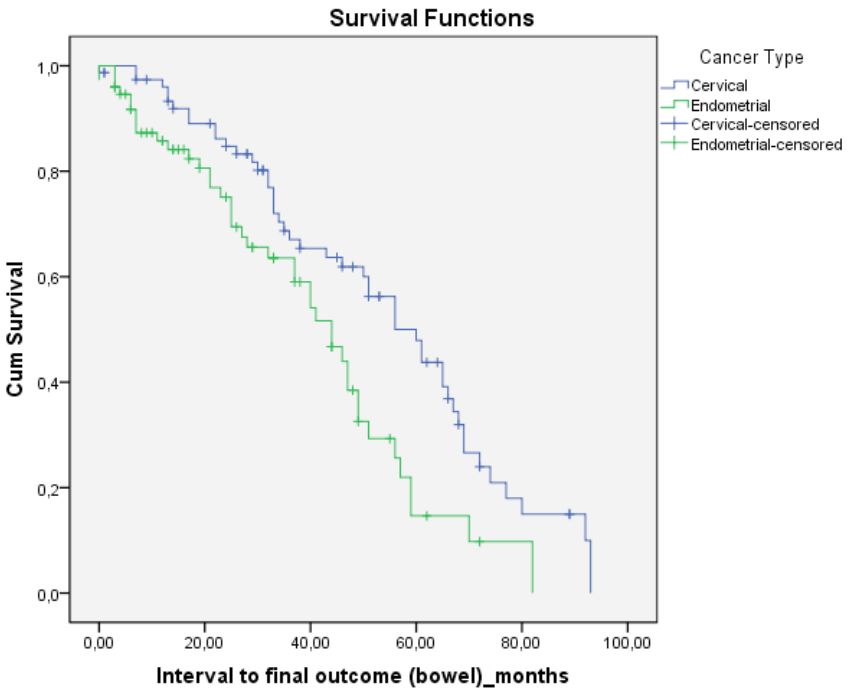


Figure 2.2. Follow-up of patients with bowel symptoms.

In univariable ordinal logistic regression analysis (Table 2.5), significant predictors of increasing severity of symptoms (from presentation to bowel status at last follow-up) were age, cancer type (cervical cancer; concurrent chemo-radiation), bowel injury requiring surgical intervention, and symptom cluster/factor 3 i.e. patients presenting with predominantly bloating, flatulence, abdominal pain, faecal urgency, per-rectal bleeding (PRB), and per-rectal mucus (PR mucus).

Variable	OR (95 % CI)	p value
Age	0.96 (0.94-0.98)	0.001
Cancer Type [Endometrial vs Cervical]	0.49 (0.26-0.93)	0.028
History of Diabetes/Hypertension	1.57 (0.64-3.96)	0.312
History of previous IBS/Diverticular Disease	1.52 (0.53-4.31)	0.436
Smoking History	0.32 (0.17-0.77)	0.008
Stage of Disease (Cervix)	0.76 (0.42-1.36)	0.356
Stage of Disease (Endo)	0.78 (0.45-1.36)	0.388
NACT pre-Chemoradiation (Cervix)	1.58 (0.41-2.50)	0.976
Extended Field RT (Pelvic Side Wall Boost/PA Strip) (Cervix)	0.37 (0.14-0.96)	0.041
Laparoscopic vs Open Hysterectomy (Endo)	0.59 (0.21-1.65)	0.311
Chemo vs no Chemo (Endo)	0.95 (0.34-2.63)	0.921
P/PA Node Dissection (Endo)	2.13 (0.58-7.75)	0.255
P/PA Node Dissection (Cervix)	0.67 (0.24-1.85)	0.442
Surgical Treatment (No vs Yes)	0.22 (0.08-0.55)	0.001
'Symptom Cluster' at presentation		
❖ Factor 1	1.18 (0.86-1.63)	0.302
❖ Factor 2	1.10 (0.80-1.51)	0.548
❖ Factor 3	1.36 (1.00-1.86)	0.053

Table 2.5. Univariate (unadjusted) ordinal regression analysis showing predictors of RIBI severity in our cohort. NACT – Neo-adjuvant chemotherapy, P/PA – Pelvic/Para-Aortic Lymph Node Dissection.

In multivariate ordinal regression analysis (Table 2.6), younger age (OR: 0.97, 95% CI: 0.94-1.0, p=0.05), smoking history (OR: 0.42, 95% CI: 0.18-0.98, p=0.044), surgical intervention (OR: 0.23, 95% CI: 0.08-0.62, p=0.005) and initial presentation with

'symptom cluster'/factor 3 (OR: 1.51, 95% CI: 1.08-2.11, p=0.017) were independent predictors of severity of bowel symptoms, after correcting for cancer type.

		95% Confidence Interval		
	OR	**Lower**	**Upper**	**p value**
Age	0.97	0.94	1.00	**0.050**
Smoking	0.42	0.18	0.98	**0.044**
'Symptom Cluster'/Factor 3	1.51	1.08	2.11	**0.017**
Cancer type (Cervical vs Endometrial)	1.21	0.55	2.63	0.637
SURGERY (No vs Yes)	0.23	0.09	0.65	**0.005**

Table 2.6. Multivariate (adjusted) ordinal regression analysis showing predictors of severe RIBI. OR – Odds Ratio.

2.3.4 Investigations and non-surgical Therapeutic Interventions for Chronic Radiation Enteritis/Proctitis in our cohort

Initial assessment of patients presenting with bowel symptoms after pelvic radiation was by the oncologists. Patients who typically had mild stable symptoms of radiation enteritis (diarrhoea alternating with loose stool, faecal urgency and increase number of motions per day (BO<4) were usually monitored with dietary manipulation advice, and anti-diarrhoeal agents Imodium and/or Codeine Phosphate prescribed. Patients were offered referral to gastroenterologists if they had symptoms that were affecting their quality of life or if symptoms worsened. Patients who presented with rectal bleeding, urgency with

faecal incontinence or any combination of symptoms which the patient deemed 'affecting quality of life' were immediately referred to a gastroenterologist.

Routine bloods tests to exclude anaemia and vitamin B12 deficiency were done for all patients presenting to gastroenterology. The most common investigation was a colonoscopy (and biopsy of any abnormal areas); 73/152 (48%) women underwent a colonoscopy whilst 22 women (14.5%) had a flexible sigmoidoscopy. Forty (40/73) of these patients had clear endoscopic evidence of distal colitis/proctosigmoiditis, and or telangiectasia/angioma attributed to radiation changes to bowel mucosa, and documented on endoscopy findings.

Radiological imaging remains part of the work-up in these women; either an MRI to exclude disease recurrence as a cause for symptoms or a CT scan, usually if bowel obstruction was suspected. Radiological imaging was useful in supporting diagnoses by demonstrating dilated and /or thickened small or large bowel loops and radiation –related strictures, as well as bladder wall and rectal thickening and oedema. Endo-anal ultrasound scans and ano-rectal physiology testing were performed to exclude sphincter defects in women who presented with incontinence/leaking. From 2010, a small proportion of patients had a SeCHAT scan to exclude bile acid malabsorption as a potential cause for ongoing diarrhoea, whilst 14 women required a hydrogen breath test to exclude small bowel bacterial overgrowth.

We analysed investigations relating to the 3 presenting symptom clusters/factors derived from factor analysis. There was a significant difference $p<0.05$ in the proportion of patients presenting with symptom cluster/factor 1 (obstructive) who went on to have an MRI/CT compared to those who scored higher for factors 2 and 3. Patients who had an endo-anal ultrasound and ano-rectal physiology as part of work-up for presenting symptoms of bowel toxicity were more likely to have scored for factor 2 compared with factor 1/3; ($p<0.001$). There was no significant difference between the 3 symptom clusters/factors for patients who underwent flexible sigmoidoscopy or colonoscopy.

The use of anti-motility/anti-diarrhoeal drugs was the most common initial intervention in most patients – 62.5% o all patients needed Imodium either regularly or as required for

a period. Only 10 patients had documented evidence of requiring Codeine phosphate as well as Imodium to control diarrhoea/frequency of motions. The trial of interventions varied over time and clinician, and was individualised based on symptoms and patients investigation results. Before 2005, some patients received trials of steroid treatments; predsol enema, predfoam and hydrocortisone suppositories.

Other treatments used of the treatment period in this study included: mebeverine (n=3), activated charcoal (n=2), asacol suppositories (1), Colesevalam and Cholestyramine when bile acid malabsorption was suspected (n=6), trial of antibiotics when small bowel bacterial overgrowth was diagnosed (n=5), biofeedback to manage faecal incontinence (n=5), and hyperbaric oxygen therapy under a clinical trial (HOTII Trial) (n=3). Thirty-two (32) patients in total (16 each in both cancer groups) required Argon Beam Coagulation (APC) therapy and colonoscopy to manage rectal bleeding; 8 of these patients required multiple procedures to control bleeding. Four (4) women were treated for transfusion- dependent rectal bleeding, with repeat blood transfusions and argon beam coagulation.

2.3.5 Surgical Management of Radiation Induced Bowel Injury

Table 2.7 illustrates the type of surgical treatment received by the women in our cohort who suffered severe late toxicity. Twenty (20/152; 13.2%) required surgical intervention to manage severe late radiation-induced toxicity. Four (4) women required (ongoing) home parenteral nutrition (TPN) after surgery to maintain nutrition, whilst 7 women still had ongoing symptoms affecting their quality of life, even after surgical resection/bypass. One patient was treated with repeated sigmoid dilatation (2006) for symptoms of intermittent sub-acute bowel obstruction with subsequent resolution of symptoms.

Surgical Procedure	Cervical Cancer	Endometrial Cancer
	n	n
Small Bowel Resection + Adhesiolysis	**7**	**2**
➢ Primary Anastamosis	2	2
➢ Ileostomy	1	0
➢ Reversal of Ileostomy	4	0
Ileocaecal Resection	2	1
Right Hemicolectomy	**4**	**2**
➢ Primary Anastamosis	2	1
➢ Colostomy Formation	2	1
End Colostomy Formation (Bypass)	**3**	**0**
Adhesiolysis only*	**1**	0
Sigmoid Stricture Dilatation	**1**	0

Table 2.7. Surgical Management of Radiation-induced Bowel Injury *Previous small bowel resection and re-obstruction

On univariate analysis, only cancer type (cervix) was associated with an increased risk of requiring surgery. There was no significant association found with tumour stage, smoking, past history of irritable bowel syndrome (IBS), divertucular disease, past history of diabetes or hypertension, the use neo-adjuvant chemotherapy, extended field radiation with pelvic side wall +/- a para-aortic boost.

In endometrial cancer patients, type of primary surgery received; open vs laparoscopic, or whether patients received adjuvant chemotherapy prior to radiation or not were also not found to be significant predictors of severe late toxicity in our cohort. Although smoking was not found to be a statistically significant predictor of severe late toxicity requiring surgery, of 6 women reported heavy smoking on diagnosis >20/day; 5/6 of these women required surgical resection of radiation- damaged bowel whilst the one remaining patient

had ongoing severe symptoms of enteritis and required surgery to repair a radiation-induced femoral occlusion.

2.3.6 Other non-bowel radiation-induced toxicity in cohort (Table 2.9)

	Cervical Cancer		**Endometrial Cancer**	
	n		n	
Radiation Cystitis	9*		3	
Colo-Vaginal Fistula	1		0	
Recto-Vaginal Fistula	2		0	
Vesico-Vaginal Fistula	2		0	
Radiation-induced Ureteric Stenosis	2		0	
Radiation-induced Femoral Occlusion	1		0	
Avascular Necrosis Femoral Head	1		1	
Radiation-induced Osteonecrosis	1		0	
Radiation-induced Vaginal Necrosis	1		0	
Vaginal Stenosis	1		0	
Total	**21**		**4**	

Table 2.9. Non-bowel related toxicity in our cohort

2.4 Conclusions and Discussion

The aim of this study was to describe and analyse the nature of presenting symptoms of bowel injury thought to be directly related to radiation treatment in our cohort of women and to determine associations with severity and chronicity of symptoms. Data was

clinician-reported and collected retrospectively from patient notes. It is important to acknowledge not only that the reported number of cases and the proportion of women who seek help are only a fraction of true prevalence (Gami et al, 2003; Andreyev et al, 2005), but also that this still, underestimates the actual frequency of pathologic changes and 'bowel injury', given some women may be asymptomatic and symptoms do not always correlate with disease activity directly related to radiation changes.

Studies (Khalid et al, 2006; Olopade et al, 2005) using patient-reported symptom tools; validated/modified questionnaires have shown this method of collecting toxicity data to be more sensitive in characterising symptoms than clinician-based reporting without toxicity scores/questionnaires. Yet others (West and Davidson, 2009) acknowledge, that outside of the clinical trial setting these questionnaires/scoring tools (especially the CTCAEv3), are impractical in the clinical setting. Barraclough et al, (2012), in a prospective analysis of patient-reported late toxicity in gynaecological cancers (73% cervix), had 126/226 (56%) patients withdraw from their study, with 60 patients discontinuing completion of questionnaires at various points throughout the study.

We appreciate that it is always difficult to make concrete conclusions from retrospectively collected data, yet this summary of our centre's experience gives some insight into current practice and highlights the significant proportion of women who suffer symptoms affecting their quality of life persisting with varying severity and chronicity. What is clear is that patients present with 'clusters' of symptoms and this data, and if collected prospectively and systematically at oncology follow-up, over time points, may well provide further evidence of the clinical course and predictors of severity of bowel symptoms.

We demonstrated the nature of symptoms reported and recorded in our cohort and found faecal urgency to be the most common reported (and documented) symptom, in keeping with the literature (Gami et al, 2003; Denham et al; 1999, Andreyev et al, 2010; Putta and Andreyev, 2005). Multivariate analysis in our study showed that younger age, treatment for cervical cancer and presenting with the multiple symptom 'cluster' that included; *bloating, flatulence, abdominal pain, faecal urgency, rectal bleeding and rectal mucus* were associated with more severe ongoing symptoms at follow-up (median 34.5 months).

Capp et al (2009) have best defined symptom clusters for rectal toxicity in men treated for prostate cancer with data obtained through modified self-assessed questionnaires, and follow-up at 1, 2, and 3 years after radiotherapy. This study highlights the flaws within the CTC (common toxicity criteria) grading scale, especially in identifying rectal injury. The clusters identified (made up of only 8 individual components/symptoms, at different time points, and as we have done, showing faecal urgency to be at the 'core' of all symptom clusters. What remains unclear is how the various symptom clusters identified related to underlying radiation-induced patho-physiological processes.

Barraclough et al (2012) in their study, use factor analysis to identify the most important questions likely to account for inter-patient variability in subjective toxicity using a disease site-specific questionnaire developed from the LENT-SOMA scales which was used to score patient data. Both studies highlight the need for a better guide for clinicians in assessing patients at initial presentation (and prior to radiotherapy). It is our belief that more data available to test the reproducibility of the symptom cluster approach and the degree of severity of each symptom may well help to better identify the type and site of the underlying radiation-induced bowel injury pathological processes and thus guide management.

I demonstrated a prevalence of around 13% of women in our cohort with severe late bowel injury requiring surgical intervention, highlighting the associated morbidity and the need for further research to try to identify these patients at risk and offer early interventions. I recognise a number of limitations in our study. All data collected was performed entirely by me to ensure consistency of record reviews. All relevant correspondence, letters, investigations and results related to each patient were reviewed. Patients with incomplete or no follow-up documentation were excluded (n=22).

Collecting clinician-reported data retrospectively relies on documentation of different clinicians with varying views of symptoms, experience dealing with patients' bowel toxicity, and assessment which all may be influenced by the patients' clinical situation. It is impossible to correct for under-reporting and lack of documentation especially for those symptoms that may have been deemed 'less serious' by clinicians. There is also the question of whether bowel toxicity is more likely to be reported and recorded in

'healthier' survivors. Patients with disease recurrence and progression are less likely to report symptoms which may be related to radiation-induced bowel injury as they are no longer focused on symptoms of toxicity, but rather worrying about symptoms related to their cancer progression.

As this was a retrospective study, there was no data identifying women's bowel function prior to radiotherapy, however a thorough past medical history, including any reported abnormal bowel symptoms were routinely obtained from each patient at our centre prior to treatment and recorded on a 'front sheet'. All symptoms reported at initial presentation, referral to gastroenterology and on first consultation with gastroenterology were recorded. Analysis of clinical features did not take into account the grading/severity of each symptom – for example the degree of urgency in combination with other symptoms in a 'cluster' might indeed represent different underlying patho-physiological processes for two different patients.

Prospective studies are urgently needed to better understand the natural history of radiation-induced bowel injury to guide the development of objective biomarkers of toxicity, and a standard in assessing degree of toxicity. This study also highlights the important role of all clinicians; oncologists, gynaecologists and referral to gastroenterologists in the follow-up and management of treatment related morbidity.

Chapter Three **A Clinical Score to Predict Severity and Progression of Radiation-induced Bowel Injury (RIBI)? A potential means to improve reporting of symptoms after Pelvic Radiotherapy in Cervical and Endometrial Cancers.**

Chapter Three A Clinical Score to Predict Severity and Progression of RIBI?

3.1 Measurement Tools in Clinical Oncology: Background and Introduction

The identification of patients at risk of significant gastrointestinal toxicity after radiation treatment for gynaecological cancers in clinical practice remains inadequate. The overall morbidity attributed to radiation-induced bowel injury (RIBI) remains unknown as studies measuring delayed toxicity after treatment for cervical and endometrial cancers are mostly incomparable. A universal, acceptable scoring tool that is practical in the clinical setting is essential. In the context of increasing survivors, the pressures of follow-up consultation times, the collection of reliable toxicity data must be improved.

Historically, the Radiation Therapy Oncology Group and the European Organization for Research and Treatment of Cancer (RTOG/EORTC) scale (Cox et al, 1995) was used to measure toxicity in clinical trials. The Late Effects on Normal Tissues – Subjective, Objective, Management and Analytic (LENT-SOMA) scale, the first to include both clinician (objective) and patient (subjective) scoring of toxicity is known to be difficult to use and requires a time-consuming structured interview with patients; Routledge et al (2003) report 89% compliance).

The Inflammatory Bowel Disease Questionnaire - Bowel Subset (IBDQ-B) and Vaizey Incontinence questionnaires (which incorporates important questions missing from the IBDQ_B; urgency, incontinence), when compared to LENT-SOMA has been shown to be more reliable in collecting bowel toxicity data (Olopade et al, 2005). The authors suggest this to be a more sensitive indicator of late effects than the LENTSOMA (Khalid et al, 2006). Others report the IBDQ-B in the trial setting as a reliable measure (Davidson et al, 2003; Wedlake et al, 2010)

The Franco-Italian Glossary first described by Chassagne et al (1993) and validated for use in cervical cancer (Sinistrero et al, 1993), has shown correlation with scores obtained using the LENT SOMA system (Davidson et al, 2003). The National Cancer Institute's

Common Toxicity Criteria (CTCAE version 3.0) published in 2003 (Trotti et al, 2003), and revised in 2010 (CTCAEv4.03) remains the current standard for used for monitoring (acute) toxicity in clinical trials. Other institution based tools such as the Royal Marsden Hospital scale has been shown to be useful in clinical trials in prostate cancer (Dearnaley et al, 2007).

West and Davidson (2009) reviewed the use of measurement tools for reporting gastrointestinal toxicity and highlighted the need for a scoring system both patient- and clinician-reported data that can be implemented in clinical practice. Most oncologists refer less than 50% of patients they see, with most referring less than 10%. Henson et al, (2012) sent a questionnaire to 314 clinical oncologists in the UK who treat pelvic malignancies. With a 61% response rate, the authors found most oncologists (76%) screen for bowel dysfunction after pelvic radiotherapy through history taking rather than using formal tools, and concluded that symptoms were poorly recognized, with inadequate service provision for diagnosing and managing patients with radiation – induced bowel injury and 'pelvic radiation disease'.

It is clear that current available tools are not being utilized in the clinical setting. A number of women with 'mild –moderate' disease will go on to have more significant delayed symptoms when they well might have benefitted from earlier referral. Bentzen et al (2007) document the challenges in recording, analyzing and reporting toxicity data and highlight that late effects may persist or even progress in severity affecting long-term quality of life and potentially compromise survival benefit.

In this chapter, I will attempt to create a scoring tool using revisited data from chapter 1. It remains to be shown in prospective studies whether the questionnaire approach is relevant in context of diagnostic pathways and helping to identify the specific patho-physiology of the bowel injury. I propose collecting and analyzing data based on the 'cluster' of symptoms patients present with.

3.2 Materials and Methods

Study Cohort

I revisited data obtained from my retrospective study as described in chapter 2. Clinical features at presentation with radiation-induced bowel injury (RIBI) after treatment for cervical and endometrial cancer was available from our retrospective cohort (Kuku et al, 2013). This included the 152 women diagnosed with mild to severe RIBI between February 2003 and June 2010 within the North London Cancer Network (UCLH). Clinician-reported toxicity data was obtained from records was utilized for this analysis. All patients were identified as presenting with new bowel symptoms 3 months after completion of radiotherapy.

Cervical cancer patients received concurrent weekly Cisplatin along with primary radiotherapy (50.4Gy in 28 fractions over 5.5 weeks using 8-15mV photons) and intracavity brachytherapy using an ovid system (15Gy in 2 fractions/HDR/point A). When used, extended fields; 45Gy in 25 fractions over 5 weeks and a further 5.04Gy in 3 fractions delivered to the pelvic side wall (n=27). Endometrial Cancer patients received 45Gy in 25 fractions with an additional 12Gy in 2 fractions to the vaginal vault. When adjuvant treatment was given, patients received 6 cycles of Carboplatin AUC 5 and Paclitaxel 175mgmin^{-2} every 3 weeks.

Exploratory and Confirmatory Factor Analysis

The cohort was randomly divided into two subsamples to create a 'Test set' and 'Validation set'. We performed an exploratory factor analysis (EFA) of the 14 items (symptoms/signs) on the first subsample (test set) to prove the validity of the 3-(factor/cluster) structure. The EFA was then applied to the second subsample (validation set) to determine whether the proposed clustering structure from the first subsample was maintained. Patient demographics for the two subsets are shown in Table 3.1; there were no statistical differences between the two groups.

Variable	EFA 'Test Set' (n=75)		CFA 'Validation' Set (n= 77)	
	N	**%**	**N**	**%**
Cancer Type				
Cervical	44	58.7	33	42.9
Endometrial	31	41.3	44	57.1
Smoker				
No	53	70.7	51	66.2
Yes	17	22.7	20	26.0
Ex-Smoker	5	6.6	6	7.8
Stage				
Stage I	18	24.0	25	32.5
Stage II	41	54.7	33	42.9
Stage III	11	14.7	17	22.1
Stage IV	5	6.6	2	2.6
Histology				
SCC	32	42.7	25	32.5
Adenocarcinoma	35	46.7	42	54.5
Other	8	10.6	10	13.0
Previous Bowel History*				
No	67	89.3	69	89.6
Yes	8	10.7	8	10.4
Diabetes or Hypertension				
No	63	84.0	64	83.1
Yes	12	16.0	13	16.9
Chemotherapy				
No	50	66.7	46	59.7
Yes	25	33.3	31	40.3

Table 3.1. Patients Demographics. There were no statistical differences between the groups for any of the variables (Chi-squared test).

Following confirmation of the stability of the clustering using confirmatory factor analysis (CFA), I used factor loadings from each item (symptom/sign) to create score points to represent the weighting of each item within a factor/cluster. To measure and

compare the predictive accuracy of the model in the test and validation sets, we generated 'receiver operating characteristics' (ROC) curves and compared their C-statistics (AUC). A template for a final proposed RIBI-Clinical Score was generated using factor loading score points. SPSS version 21 - IBM was used for all statistical analyses.

3.3 Results

3.3.1 Statistical Analysis

Validation

The EFA suggested a 3 factor solution – 1st factor – 4 items, 2nd factor – 4 items, 3rd factor – 5 items, although results suggested that 'PR Mucus' should be dropped (Communality 0.06) and loading values lowest (0.06 – 0.179). This was not surprising, given the small number of patients in this cohort who were documented to have presented with this symptom (8.6% of all patients only).

Subsample 1: Exploratory factor analysis

This first subset included 75 women (mean age = 57.3, range 22-85]. Other demographic information is shown in Table 3.1 above. The 14 items of the RIBI score were subjected to principal components analysis (PCA) and revealed the presence of three components, with Eigen values exceeding 1, explaining 23.9%, 18.1%, and 14.1% of the variance respectively. An inspection of the scree plot revealed a clear break after the third component. Using Cattell's (1966) scree test, it was decided to retain three components for further investigation. The three-component solution explained a total of 56.1% of the variance. Factor loadings for all three components are shown in Table 3.2. To aid in the interpretation of the three components, Oblimin Rotation was performed.

	Pattern Matrix			Structure Matrix			Communalities
	Component			Component			
	1	2	3	1	2	3	
NAUSEA	**0.932**	-0.056	0.084	**0.927**	-0.004	0.057	0.868
VOMITTING	**0.932**	-0.056	0.084	**0.927**	-0.004	0.057	0.868
SABO	**0.755**	0.021	0.249	**0.75**	0.07	0.23	0.625
ABO	**0.64**	0.02	-0.056	**0.642**	0.051	-0.072	0.416
BO>4D	0.016	**0.904**	-0.11	0.066	**0.9**	-0.075	0.78
BO<4D	-0.233	**-0.812**	0.242	-0.281	**-0.815**	0.217	0.823
INCONTINENCE	-0.225	**0.649**	0.021	-0.192	**0.638**	0.053	0.458
DIARRHOEA	0.029	**0.596**	0.203	0.054	**0.605**	0.225	0.408
BLOATING	0.187	-0.014	**0.749**	0.166	0.025	**0.743**	0.588
ABDOPAIN	0.361	-0.019	**0.683**	0.341	0.026	**0.672**	0.582
PRB	-0.179	-0.192	**-0.581**	-0.173	-0.223	**-0.584**	0.413
URGENCY	-0.41	0.436	**0.507**	-0.401	0.435	**0.535**	0.626
FLATULENCE	-0.263	-0.168	**0.487**	-0.284	-0.162	**0.487**	0.339
PRMUCUS	-0.153	-0.06	**0.179**	-0.161	-0.061	**0.18**	0.06

Table 3.2. EFA from 14 items. Factor analysis used to split the items (signs/symptoms) into 3 'clusters'/components comprising groups of symptoms patients presented with.

As described in chapter 1, the factor analysis again divided the items into 3 clusters. Component/Factor 1 suggested 'obstructive' symptoms, because it maintains high loading in symptoms suggestive of (radiotherapy-induced) bowel stricture/obstruction (*nausea, vomiting, sub-acute bowel obstruction and acute bowel obstruction*). Component 2 – *diarrhoea and increased bowel frequency < 4 x daily or >4 x daily and faecal incontinence*) could suggest symptoms of small bowel dysfunction, whereas component 3 is more pathgnomonic, (though not definitive) of 'colitis-proctitis/proctopathy' symptoms (*bloating, flatulence, abdominal pain, faecal urgency, per-rectal bleeding, and per-rectal mucus).*

Subsample 2: Confirmatory Factor Analysis

The second subset consisted of 77 women (mean age = 56.8, range 28-80) and was used to re-test the factor structure of the items. Confirmatory factor analysis was used in order to test whether the proposed structure of the RIBI symptom clustering remained stable in another sample. Common model fit-measures were used: the ratio of the model's chi-square with the degrees of freedom (χ^2/df), Comparative Fit Index (CFI), (Bentler, 1990), and the Root Mean Square Error of Approximation (RMSEA) (Steiger, 1990). The path diagram for the CFA model, illustrating the individual factor loadings for each item to those in the EFA is shown in Figure 3.1.

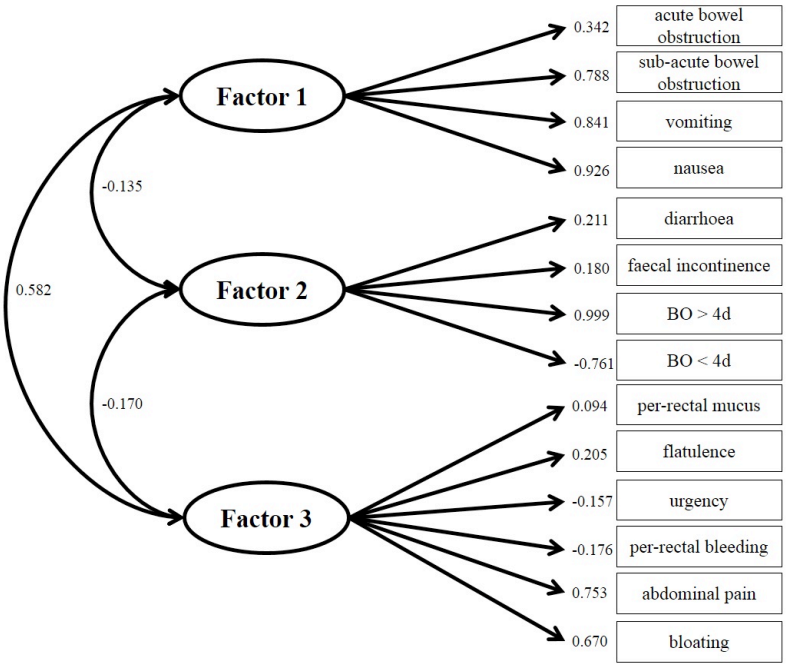


Fig 3.1. CFA. Factor Loadings comparable to EFA. The factor loadings (next to the boxes with symptom labels are compared to factor loadings from table 3.2) are a measure of the match of the clustering analysis to the test set.

Assessing the fit indices of the CFA these were acceptable with regards to comparative fit indices, the parsimony adjusted measure RMSEA and the standardized χ^2 (χ^2/df) (Table 3.3). I observed that in most cases the factors loadings of the CFA (sign and magnitude) were similar with those with from the EFA. That indicates satisfactory stability of the clustering of symptoms.

Model Fit indices	Recommended Value	Results
χ^2/df	≤ 3.0	1.190
CFI	≥ 0.9	0.966
RMSEA (90% CI)	≤ 0.08	0.050

Table 3.3. Fit indices of the CFA for Cognitive Affective Factors

(χ^2/df); the ratio of the model's chi-square with the degrees of freedom, (CFI); Comparative Fit Index, (RMSEA); Root Mean Square Error of Approximation

3.3.2 Developing a scoring system taking into account factor loadings/symptom clusters

The validity of the 3-factor model confirmed, I the utilized factor loadings from the total sample size of 152 patients as described in chapter 1. Factor loadings for each item within a cluster were multiplied by 2 to obtain a whole number nearest to 1, with the closest integer used as a simple score 'point' (-1 to 2) (Table 3.4).

Symptom	Factor loadings			Points
	F1	F2	F3	
Nausea	**0.889**			**2**
Vomiting	**0.869**			**2**
SABO	**0.817**			**2**
ABO	**0.547**			**1**
BO>4d		**0.915**		**2**
BO<4d		**−0.847**		**-2**
Diarrhoea		**0.545**		**1**
FI		**0.507**		**1**
Bloating			**0.695**	**1**
Flatulence			**0.589**	**1**
Abdo pain			**0.546**	**1**
Urgency			**0.460**	**1**
PRB			**−0.339**	**-1**
PR mucus			**0.339**	**1**

Table 3.4. Score Points using Factor Loadings

Four scores were generated for each patient: a score for each factor/cluster, and a total score. This takes into account the weighting of all 14 signs/symptoms each patient presented with, multiplied by the 'loading' or 'weighting' within each factor /cluster. ROC analysis was performed to compare the diagnostic accuracy of each factor/cluster score between resolved/mild disease and moderate to severe disease. Figure 3.2 demonstrates the ROC curves generated to test and compare the predictive accuracy of the scoring model for the entire data set.

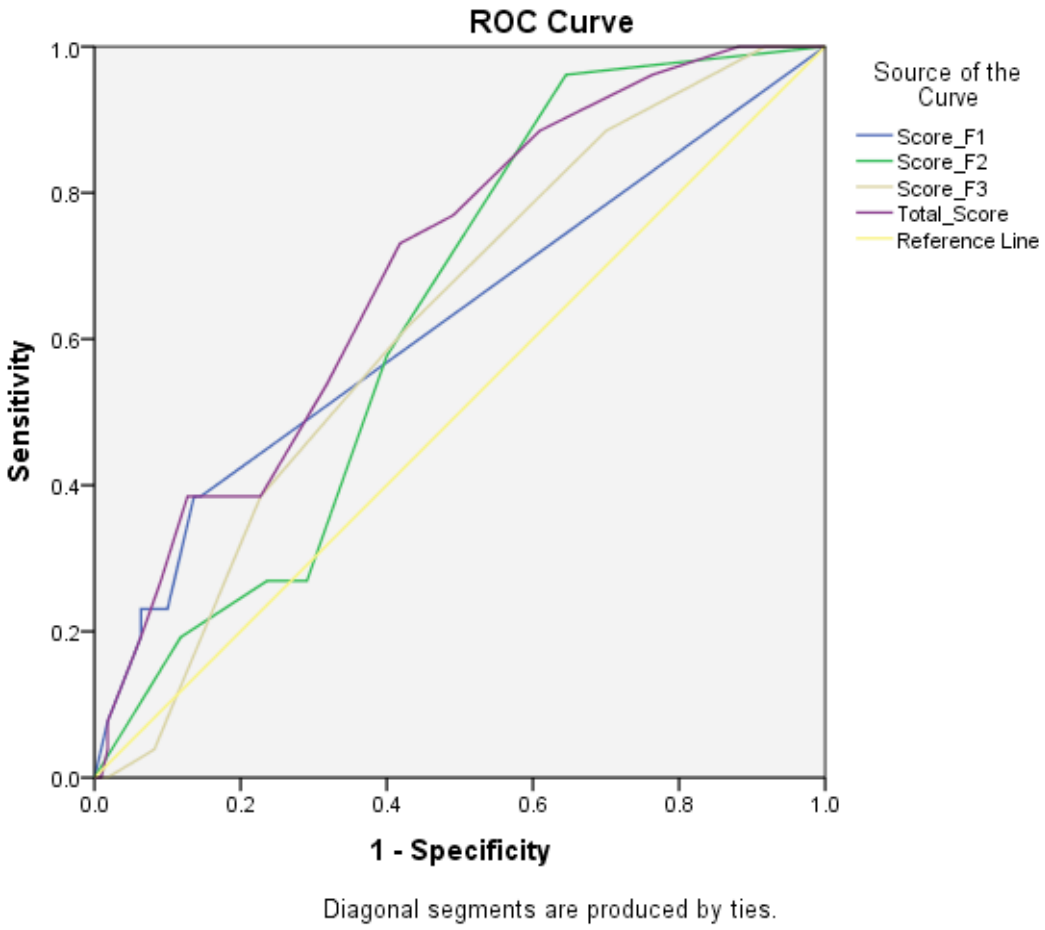


Figure 3.2. ROC analysis comparing the diagnostic accuracy of each symptom cluster/factor score; resolved or mild ongoing bowel symptoms cf ongoing moderate to severe disease. Results are shown in Table 3.5 below.

The calculated AUC values for the scores in the 3 factors (clusters) and the total score are shown in Table 3.5 with confidence intervals.

The total score and score for F2 (Factor/Cluster 2) had the highest diagnostic accuracy; AUC 0.697 (95% CI 0.593 to 0.802) and AUC 0.636 (95% CI 0.535 to 0.738) respectively. All were moderately satisfactory (AUC >0.600). The various cut-off scores with their sensitivity and specificity are shown in Table 3.6.

Area Under the Curve					
Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Score_F1	.622	.066	.054	.492	.751
Score_F2	.636	.052	.031	.535	.738
Score_F3	.624	.056	.050	.515	.733
Total_Score	.697	.053	.002	.593	.802
The test result variable(s): Score_F1, Score_F2, Score_F3, Total_Score has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.					
a. Under the nonparametric assumption					
b. Null hypothesis: true area = 0.5					

Table 3.5. ROC Analysis for scores for each Symptom Cluster/Factor (F1,F2,F3)

Test Result Variable(s)	Positive if Greater Than or Equal To	Sensitivity	Specificity
Score_F1	-1	100	0
	0.5	38.5	85.5
	1.5	38.5	86.4
	2.5	26.9	89.1
	3.5	23.1	90
	4.5	23.1	93.6
	5.5	19.2	93.6
	6.5	7.7	98.2
	8	0	100
Score_F2	-3	100	0
	-1.5	96.2	35.5
	-0.5	57.7	60
	1	26.9	70.9
	2.5	26.9	76.4
	3.5	19.2	88.2
	5	0	100
Score_F3	-2	100	0
	-0.5	100	8.2
	0.5	88.5	30
	1.5	61.5	57.3
	2.5	38.5	77.3
	3.5	3.8	91.8
	4.5	0	98.2
	6	0	100
Total_Score	-4	100	0
	-2.5	100	5.5
	-1.5	100	11.8
	-0.5	96.2	23.6
	0.5	88.5	39.1
	1.5	76.9	50.9
	2.5	73.1	58.2
	3.5	53.8	68.2
	4.5	38.5	77.3
	5.5	38.5	87.3
	6.5	26.9	90.9
	7.5	19.2	93.6
	8.5	7.7	98.2
	9.5	3.8	98.2
	11	0	99.1
	13	0	100

Table 3.6. Cut off scores of ROC analysis with respective sensitivity and specificity

For this cohort, a total score cut-off of 5.5 confers a sensitivity of 38.5% and specificity of 87.3%. As a predictive screening tool, ensuring a low number of false negatives, rather than false positives would be more useful when collecting toxicity data. Increasing the study numbers when validating this tool on a prospective cohort would improve both sensitivity and specificity.

3.3.3 Creating a Radiation- Induced Bowel Injury Score (RIBI) Score

I created a 14-item score based on the above analysis. This ideally would also include weighting for the degree (grade) of each symptom based on frequency of experiencing these symptoms. The score developed (Template shown as Table 3.7) is a multiple point score that can potentially be modified further to incorporate other variables.

It is important that any score takes into account the degree to which symptoms affect a patient's quality of life. While recognizing that mild-moderate urgency may affect two different patients in different ways, some degree of uniformity can be achieved by eliciting symptom frequency, i.e. how often a symptom occurs – daily, weekly, monthly etc.

Each symptom cluster score would highlight the predominant 'cluster', with the total score taking into account all signs/symptoms. The multiplying integers represent the relative weighting within the cluster (note bowel frequency <4 has a factor scor of (-2), whilst bowel frequency >4/day has a factor score of (+2).

Grade/ Severity	0	1	2	3	4	Factor Score	Total
Nausea						2	
Vomiting						2	
SABO*						2	
ABO**						1	
BO>4						2	
BO<4						-2	
Diarrhoea						1	
Faecal Incontinence						1	
Bloating						1	
Flatulence						1	
Abdo pain						1	
Urgency						1	
PRB						-1	
PR Mucus						1	
Total Score							

Table 3.7. 'RIBI' Score Template: Radiation Induced Bowel Injury after Pelvic Radiotherapy; UCLH Departments of Oncology, Gastroenterology & Nutrition. Validation required on a prospective cohort with grades of severity for each item. For grading of each item; 0 : none, not experienced, 1: occasional (rarely), 2: intermittent (i.e. once/twice monthly), 3: persistent i.e. weekly and 4: refractory (i.e. daily)

3.4 Conclusion and Discussion

It is well recognized (Henson et al, 2012) that the use of the available formal tools to report toxicity data by oncologists and service-provision in the UK for patients presenting with symptoms of radiation-induced bowel injury, or 'pelvic radiation disease' is poor. Few studies have addressed this issue although several research groups in the UK are currently conducting studies.

This experimental study aimed to design a tool that might help predict patients in need of early referral to gastroenterologist, whilst giving diagnostic clues that may well prevent expensive (and invasive) tests in all patients. Capp et al (2009) first describe this clustering process in men treated for prostate cancer. Urgency was found to be at the 'core' of many symptom clusters (made up of 8 items only), with 'bleeding' identified as a 'peripheral' symptom. This research group suggests that the 'position' of symptoms in the cluster may be related to the path-physiological process. They also highlight the shortcomings in the CTCAEv3.0 in failing to define rectal injury especially as it is well documented that faecal urgency is the most common and troubling symptom affecting men and women after radiation (Andreyev 2007).

Prediction and early recognition of patients with RIBI could improve the quality of life in these women if managed appropriately. Multivariate ordinal regression analysis in chapter 1 suggested that patients presenting with symptom factor/cluster 3; (bloating, flatulence, abdominal pain, faecal urgency, per-rectal bleeding, and per-rectal mucus) were more likely to suffer severe, more progressive and chronic disease. I applied ROC analysis to evaluate the validity of our predictive scoring model and showed AUC>0.600 for each factor score and the total cores. This study is limited as it uses data collected retrospectively from a relatively small number of patients (n=152). The model needs to be tested prospectively on a larger cohort of patients from multiple centers to provide further data to substantiate its applicability, and validate the score. The development of a simple RIBI Score Online Calculator to collect data at different time points during oncology follow-up that may provide a platform for prospective data collection which

could improve individual patient care but also provide a simple, accessible tool in planning future clinical trials.

In conclusion I propose a clinical score validated in our cohort of patients that may be used to predict severity and progression of symptoms of radiation-induced bowel injury (RIBI). The score enhances prediction of severity by taking into account the weighting of each symptom within a cluster of presenting complaints and may help identify those patients needed early referral to a gastroenterologist and early intervention to prevent progression of the 'disease' process. The score is potentially easy to use in the clinical setting and could also be used as a data collection tool at sequential points during follow-up after radiotherapy for use in prospective studies to improve further research into the patho-physiology of radiation-induced bowel injury.

Part II Biomarkers in Clinical Oncology

Chapter Four Cell-cycle Markers in Cervical Cancer – utility as a predictive and prognostic marker of (chemo)-radiosensitivity and tumour response?

Chapter Four Cell-cycle Markers in Cervical Cancer – utility as a predictive and prognostic marker of (chemo)-radiosensitivity and tumour response?

4.1 Replicating Licensing Factors and The Cell-Cycle

The dysregulation of mechanisms that control cellular proliferation, differentiation and apoptosis has long been recognised as the mechanism by which cancer cells acquire growth advantage over non-cancerous cells. The DNA replication-licensing pathway consists of a complex of initiator proteins, which bind and open the DNA at origins. Replication licensing factors (RLFs) ORC, Cdc6, Cdt1, and Mcm2-7, during late mitosis and in the rest phase before DNA synthesis (G1) (Figure 4.1), assemble into pre-replicative complexes, which render replication origins 'licensed' for DNA synthesis.

During DNA synthesis (S phase), Cdc7 kinase and cyclin-dependent kinases induce a conformational change in the pre-replicative complex, resulting in recruitment of additional initiator proteins that collectively promote DNA unwinding and recruitment of DNA polymerases (Bell et al 2002; Eward et al 2004).

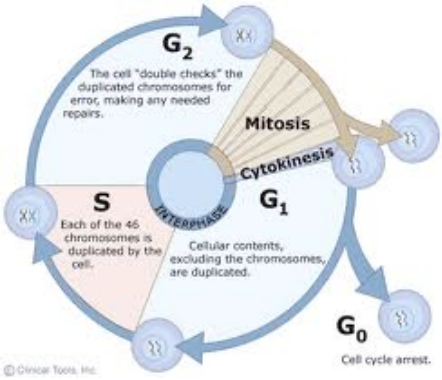


Figure 4.1. The cell-cycle. G1 and G2 shown as rest-phases between DNA synthesis (S) and mitosis (M). Cells leave the cell-cycle at G0.

4.1.1 MCM Proteins

The Mcm (minichromosome maintenance) proteins (Mcm2-7), constituents of a DNA replicative helicase, are expressed throughout the cell cycle (G1-S-G2-M) but are tightly downregulated during exit into out-of-cycle (G0) or differentiated states (Stoeber et al 2001; Barkley et al, 2007; Williams and Stoeber, 2007). The expression levels of Mcm proteins therefore reflect the proliferative capacity of a cell and they thus have been shown to be useful for cancer detection and prognosis in many tumour types (Williams and Stoeber, 2007; Baldwin et al, 2003). Williams et al, (1998) have also showed the potential use of Mcm5 in detecting low and high-grade dysplastic lesions in abnormal cervical smears.

4.2. Ki67 (Clone MIB-1)

The cell-cycle antigen Ki67 (clone MIB-1) is a cellular marker of proliferation present during all active phases of the cell-cycle (G1-S-G2-M) but absent from resting cells (G0). The precise function of Ki67 is uncertain, however the combination of its tight cell-cycle phase regulation and a short half-life have established the protein as a robust marker for proliferating cells (Yerushalmi et al, 2012).

4.3 Geminin

During S-G2-M phases, the presence of the licensing repressor protein Geminin prevents inappropriate reinitiation events at origins that have already been activated, through its interaction with Cdt1, resulting in a block to Mcm2-7 loading to chromatin (Hook et al, 2007). Geminin, therefore is used as a biomarker of S-G2-M progression.

4.4 Use of Cell-cycle markers in Clinical Oncology

Expression profiles of Mcm, together with Ki67 and Geminin measure cell cycle kinetics and allows cells in out-of-cycle states to be distinguished from those residing in cycle and can assign cells to G1 and S-G2-M phase. (Kulkarni AA et al, 2007; Loddo et al, 2009). The Mcm2/Ki67 ratio defines the proportion of cells that are licensed to proliferate, i.e. Mcm2-7 also identifies non-cycling cells with proliferative potential; the higher the Mcm2/Ki67 ratio, the greater the proportion of cells that reside in a licensed non-cycling state. (Williams and Stoeber, 2007; Kulkarni et al, 2007; Loddo et al, 2009; Dudderidge et al, 2005). Ki67, since present throughout the cell cycle in proliferating cells, together

with Geminin can be used to determine the rate of cell-cycle progression - the Geminin/Ki67 ratio may be used as an indictor of relative length of G1 phase.

Multiparameter analysis of these DNA replications licensing factors (RLFs) - Mcm2-7, Ki67 and Geminin, readily detectable by immunohistochemistry in surgical tumour biopsies have been linked to tumour cell cycle state and clinical outcome in penile squamous cell carcinoma (Kayes et al, 2009). In this study, analysis of RLF expression showed accelerated cell-cycle progression was significantly associated with high grade tumour, increased tumour size, depth of invasion and shorter overall survival time, suggesting that RLF analysis may be used as prognostic and predictive biomarkers. In this chapter, I sought to study the cell cycle kinetics in a cohort of cervical cancers and identify any potential correlation between protein expression levels of these RLFs with tumour differentiation, stage of disease at presentation, and response to chemo-radiation.

4.5 Materials and Methods

4.5.1 Study Cohort.

All patients were part of a multicenter single arm phase II study (Cervix II) (McCormack et al, 2013) of weekly neoadjuvant carboplatin and paclitaxel chemotherapy followed by standard radical chemo-radiation with cisplatin for locally advanced cervical cancer. Patients received dose-dense carboplatin (AUC2) and paclitaxel (80mg/m2) weekly for 6 cycles followed by chemo-radiotherapy with 40mg/m2 of weekly cisplatin. Radiation (with concomitant cisplatin) to the whole pelvis was given to a total dose of 50.4Gy in 28 fractions over 5.5 weeks using 8-15 mV photons. Intra-cavity brachytherapy was given following completion of external beam radiation; patients received a total dose of 15Gy in 2 fractions. Between June 2005 and October 2008, 46 patients diagnosed with squamous, adeno-squamous or adenocarcinoma of the cervix, FIGO stage IB2 – IVA were recruited to take part in the study. As part of the eligibility criteria patients had to be

age over 18, considered suitable for radical chemo-radiation, and fit for treatment. All patients had a pre-treatment biopsy during staging examination under anaesthesia (EUA). Patients who had histologically positive para-aortic lymph nodes were also included in the study.

The majority of patients were treated within the North London Cancer Network and biopsy specimens were reviewed by a specialist gynae-oncology pathologist at diagnosis. Formalin-fixed paraffin wax-embedded tissue was retrieved from the archives of the Department of Pathology (UCL Hospitals, London, UK) for 35 patients within the trial. Normal cervix tissue was obtained from patients following colposcopic assessment and biopsies. Clinical information was extracted from hospital medical records and the trials database. Ethics approval was obtained from the local research ethics committee from the joint University College London/University College London Hospitals Committees on Ethics of Human Research. Histologic grade and stage of the primary tumour were recorded. Tumour grade was defined using Broders' classification: well differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated (grade 3). Follow-up data was obtained for 35 patients with available cervical tumour tissue samples for immunohistochemistry. Table 4.1 shows the clinicopathologic characteristics of patients. The median age of all patients at time of diagnosis was 42 (range 27 – 71). Fifty-seven percent (57%) of the tumours were moderately differentiated (grade 2). Majority (80%) were of squamous cell histology.

	Frequency (%)
Age (years)	
Median (Range)	42 (23 – 71)
Grade	
1	1
2	20 (57%)
3	14
Tumour Stage	
1B2	3
IIB	23 (66%)
IIIA	1
IIIB	6
IVA	2
Histology	
Squamous Cell	28 (80%)
Adenocarcinoma	6
Adeno-squamous	1
Median follow-up (months)	37.7
Median (range)	(3.4 – 73.9)

Table 4.1. Patient Characteristics and Cancer Demographics

4.5.2 Antibodies.

Rabbit polyclonal antibody against human geminin were previously generated and validated at the UCL laboratory (Eward et al, 2004; Wharton et al, 2009). Ki67 monoclonal antibody (Mab) (clone MIB-1) was obtained from DAKO (Glostrup, Denmark) and Mcm2 monoclonal antibody (clone 46) from BD Transduction Laboratories (Lexington, KY, USA). The specificity of Ki67 and Mcm2 monocloncal antibodies were previously been confirmed in previous studies (Loddo et al, 2009; Kulkarni et al, 2007; Dudderidge et al, 2005).

4.5.3 Immunohistochemistry

Consecutive serial sections were cut from each paraffin-embedded tissue block representative of the tumour. Three-micrometer sections were cut onto Superfrost Plus slides (Leica Microsystems), dewaxed in Xylene, and rehydrated through graded alcohol to water. For antigen retrieval, slides were pressure cooked in 0.1ml citrate buffer (ph 6.0) at 103kPa for 2.5min. Tissue sections were immunostained using the Bond Polymer Define Detection Kit and Bond Polymer Define Detection kit and Bond-X automated system (Leica) according to the manufacturer's instructions.

Primary antibodies were applied at the following dilutions: Ki67 (MIB-1) (1:70), Mcm2 (1:2000) and Geminin (1:1000). Coverslips were applied using Pertex mounting medium (Cellpath). Incubation without the primary antibody was used as a negative control and tonsil epithelium was used as positive control.

4.5.4 Protein expression profile analysis

Protein expression levels were expressed as a labelling index (LI), as previously described (Dudderidge et al, 2005; Shetty et al, 2007; Kulkarni et al, 2007). Slides were evaluated at x100 magnification to view the advancing edge of the tumour and select areas with the most densely stained tumour cells starting from the edge to the centre of the tumour. Three to five areas were image captured at x400 magnification with a charge-coupled device camera and AnalySIS image analysis software (SIS).

Images were then printed for quantitative analysis, which was undertaken before I became aware of clinicopathologic and outcome (all slides were blinded by using laboratory numbers). Both positive and negative cells in the field were counted, excluding stromal and inflammatory cells. A minimum of 500 cells was counted for each case. The LI was calculated using the formula: LI = number of positive cells/total

number of cells x 100. Reassessment of 20 randomly selected cases by an independent assessor (Ian Proctor, Pathologist & laboratory Supervisor) showed concordance in all cases.

4.5.5 Statistical analysis

Biomarkers labelling indices were summarised using median and interquartile ranges. Mann-Whitney test was used to compare relationship between biomarker expression and tumour differentiation, stage of disease and type of primary tumour. Disease-free and overall survivals (DFS and OS) were measured from the date of study registration until relapse, death from any cause, or the date last seen alive. Events for DFS included recurrence or death from any cause.

All survival endpoints were measured from the date of registration, and patients who did not have the event of interest were censored at the date of last follow up. Kaplan-Meier curves and Cox regression analyses were performed. Analyses performed using STATA 12 and all p-values are two-sided. All factors were assessed separately for association with biomarker expression. All tests were two-sided and a statistical significance level of $p<0.05$ was used.

4.6 Results

4.6.1 Cell-cycle marker expression in normal and malignant cervical epithelium

Protein expression profiles for Mcm2, Ki67, and Geminin were determined in both benign and malignant lesions of the cervix using previously characterised monospecific antibodies against Mcm5 protein and Ki67 (MIB-1). In contrast to dysplastic and

malignant epithelium, all 13 control cases of normal squamous epithelium only showed protein expression restricted to the basal and suprabasal cells as previously described (Williams et al, 1998; Kayes et al, 2009); median Mcm2; 65.1% (range 34% – 98.4%) and median Ki67 (MIB-1); 11.6% (range 9%- 44.1%.

Geminin expression in normal cells were low; median 6.1% (range 0 – 10.2%) (Table 4.2). Cells in the superficial layers with well-differentiated phenotype showed a low level of Mcm expression (0-2%).

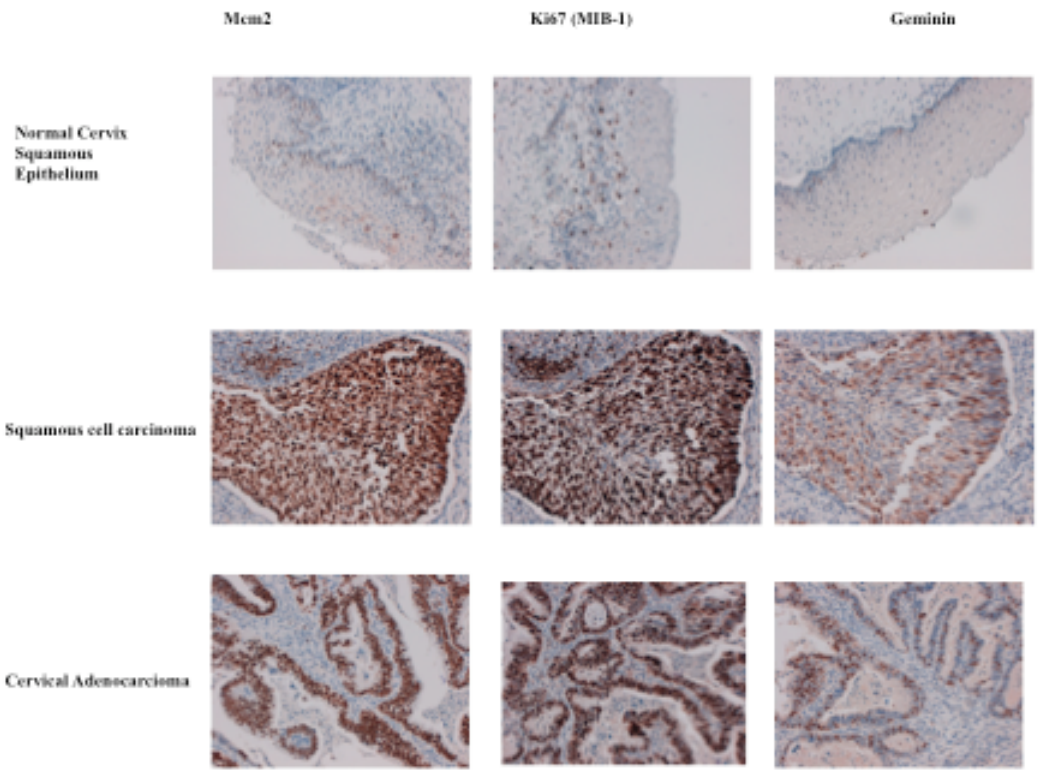


Figure 4.2. Photo-micrographs of paraffin wax-embedded tissue sections of representative normal squamous epithelium (n=13), squamous cell carcinoma-in-situ (n=28), and cervical adenocarcinoma (n=7), immunohistochemistry stained with antibodies to Ki67, Mcm2, and geminin. Magnification X200

4.6.2 Cell-cycle state and tumour differentiation, stage and survival.

Protein expression profiles for Mcm2, Ki67 and Geminin were determined in the 35 cervical tumours. No significant relationship was found between biomarker expression and clinicopathologic variables. Expression levels of Mcm2, Ki67 and Geminin were not associated with tumour grade (G1/2 vs G3), stage or overall survival. Median Mcm2 expression for G1/2: 92.2%, interquartile range (86.0 – 92.0) and G3: 89.2% (84.6 - 97.2) respectively. Median Ki67 was lower, G1/2: 76.8% (59.0 – 86.0) and G3: 77.3% (65.8 - 83.6) than Mcm2 expression, but with both biomarkers extending over a broad range. Table 4.2 summarises the statistical analysis of the relationship between marker expression and clinicopathological variables.

Median Geminin expression was lower for all cases: G1/2 (29.1%) and G3 (37%); geminin is only present during S-G2-M and represents a lower growth fraction (Eward et al, 2004). There was a trend towards a higher Mcm2/Ki67 ratio in squamous cell compared to adenocarcinoma (1.3 vs 1.0, p=0.06), but this was not statistically significant. The Mcm2/Ki67 ratio reflects the proportion of non-proliferating cells that are licensed for DNA replication and we would expect this to increase with increasing grade to reflect a shift in the proportion of cells licensed to proliferate from well differentiated to poorly differentiated tumours. There was no association with Geminin/Ki67 ratio and tumour differentiation as shown in epithelial ovarian cancer (Kulkarni et al, 2007; Loddo et al, 2009). This ratio indicates the relative length of G1 phase and the rate of cell-cycle progression.

	Mcm2	Ki67	Geminin	Mcm2/Ki67	Geminin/Ki67
Normal Cervix (n=13)	65.1 (38.9 70.6)	11.6 (10.0 –26.4)	6.3 (2.9 – 7.7)	3.40 (2.60 -4.63)	0.35 (0.13 – 0.55)
Cervical Cancer (n=35)	90	77	32	1.19	0.45
Grade					
1/2 (n=21)	92 (86-98)*	77 (59-86)	29 (23-38)	1.19 (1.05-1.51)	0.45 (0.32-0.57)
3 (n=14)	89 (85-97)	77 (66-84)	37 (27-43)	1.19 (1.08-1.41)	0.48 (0.37-0.55)
p-value**	0.50	0.50	0.14	0.80	0.48
Stage					
IB2/IIB (n=26)	91 (70-98)*	77 (56-86)	32 (26-39)	1.19 (1.05-1.51)	0.46 (0.37-0.57)
IIIA/IIIB/IVA (n=9)	88 (86-98)	78 (66-84)	36 (23-40)	1.24 (1.07-1.48)	0.43 (0.33-0.50)
p-value**	0.97	0.68	0.85	0.85	0.63
Histology					
SCC (n=28)	92 (86-97.8)*	76 (60-83)	32 (25-40)	1.25 (1.08-1.49)	0.49 (0.36-0.58)
Adenocarcinoma (n=7)	90 (51-98)	82 (46-89)	37 (25-39)	1.02 (0.85-1.19)	0.45 (0.32-0.46)
p-value**	0.43	0.43	0.93	0.06	0.38

Table 4.2. Relationship between biomarker expression and clinicopathologic variables. Labelling index (expressed as percentage) used, *Median (interquartile range), ** Mann-Whitney test.

There was no evidence of an association between cell-cycle phase specific markers and stage (IB2/IIB vs IIIA/IIIB/IVA), grade or survival (disease-free and overall survival). Figure 4.3 shows the LIs for all markers by tumour grade. There were no differences found for percentage expression and proliferation markers between well differentiated and moderately differentiated tumour and poorly differentiated (high grade) tumours.

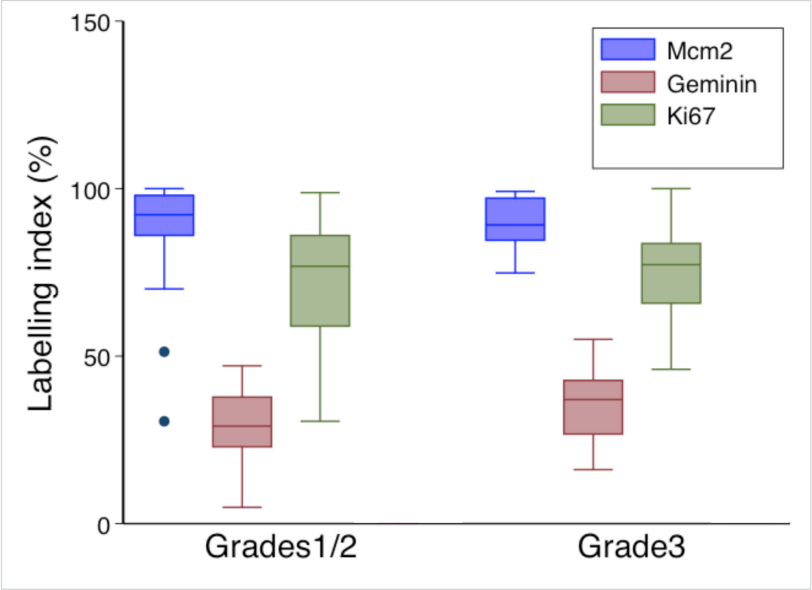


Figure 4.3. Distribution of biomarkers by tumour grade; median and interquatile ranges (boxed), and range for Mcm2, Ki67 and Geminin. There is no statistical difference shown between the groups G1/2 (n=21) and G3 (n=14)

At the time of analysis (survival data collected until December 2012 as part of a clinical trial (McCormack et al, 2013), 11 patients had relapsed and died. One patient died of a non-cancer related cause (cerebral haemorrhage). Median follow-up period was 37.7 months (range 3.4 - 73.9). Cox regression analysis was used to calculate survival time. 11(31%) of patients had died at the time of analysis. There was no association found between disease-free (DFS) and overall survival (OS) and expression of Mcm2, Ki67 and geminin on univariate analysis.

Kaplan-Meier curves were generated to show DFS and OS by grade (Figures 4.4a and b).

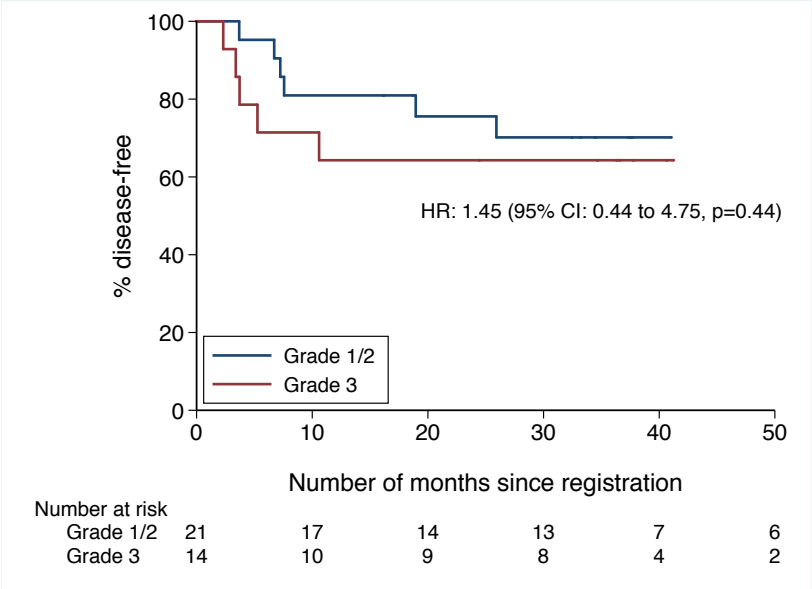


Fig 4.4a. Disease-Free Survival (DFS) by Tumour Grade

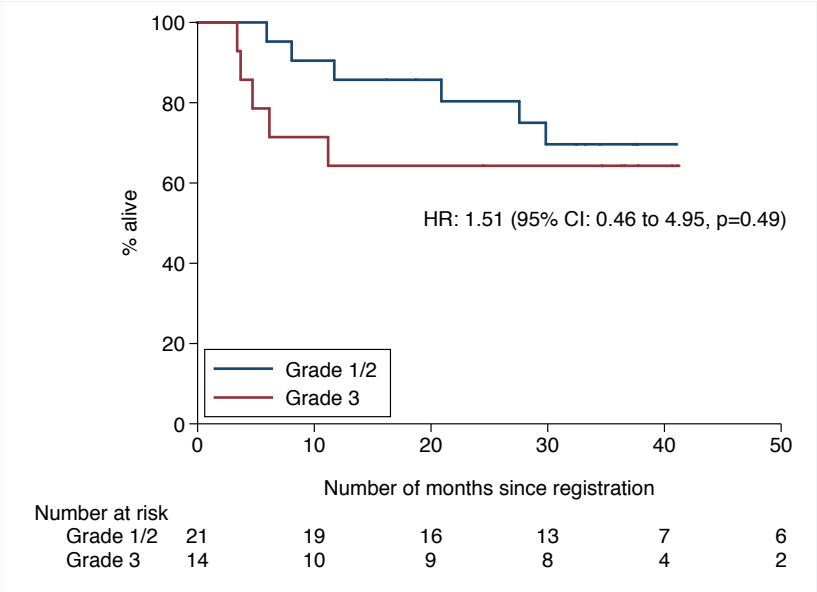


Figure 4.4b. Overall Survival (OS) by Tumour Grade

There was poorer survival (DFS and OS) in patients with G3 tumours but this was not statistically significant; DFS (G3 vs G1/2); HR 1.45 (95% CI: 0.44-4.75), p=0.44), OS; HR 1.51 (95% CI: 0.46-4.95, p=0.45).

4.6.3 Radiation toxicity – Incidence in this cohort

The recorded incidence of radiation induced bowel injury in this cohort was 20% (7/35). Three (3) out of these 7 women had significant symptoms requiring investigations. There was no difference found for labelling indices for all of the cell-cycle markers in these 7 women compared to women who did not experience or present with symptoms of radiation-induced bowel injury.

Sub-analysis in the small numbers did not show a difference in survival between these two groups and thus I was unable to support the hypothesis of the potential of cell-cycle phase specific markers as a marker of (chemo)-radiosensitivity, with an associated increased risk of normal tissue injury.

4.7 Conclusions

In this pilot study, I assessed the utility of Mcm2, Ki67 and Geminin as biomarkers in cervical cancer. In contrast to studies in other cancers, cell cycle phase specific markers do not predict disease grade or stage and were not predictors of disease progression and overall survival. In our small sample size of 35 tumours, the expression of each cell cycle biomarker was very high in all cases. All the cases of squamous cell carcinoma of the cervix (n=28) and all but one of the adenocarcinomas (n=7) in this study displayed an aggressive 'actively cycling' phenotype with high Mcm2, Ki67, and geminin expression.

Univariate analysis did not show an association between RLF expression and survival. There was no statistical difference in both disease-free (DFS) and overall survival (OS) between grade1/2 and high-grade disease. There appeared to be no association with expression of markers between low and high grade, with differentiated (G1/2) tumours expressing similar high levels of RLFs as poorly differentiated tumours (G3). We recognize our study is limited due to small numbers however our results raise the question of whether degree of tumour differentiation is an unreliable prognostic marker in cervical cancer, unlike in other epithelial tumours (e.g. ovary). The most likely explanation for this would be the viral aetiology of this disease. HPV E6 and E7 oncogenes have a wide range of cellular targets, most notably the degradation of p53 tumour suppressor protein by E6 and the abrogation of pRB tumour suppressor function by E7. Chronic expression of E6 and E7 are understood to lead to the acquisition of genomic instability and proliferative capacity.

Gradual down-regulation of Mcm2-7 occurs as cells mature and adopt a fully differentiated functional phenotype. There is evidence to show increasing mcm expression from low to high-grade dysplastic (Williams et al, 1998). The arrested differentiation that characterises cancer, particularly in high-grade tumours, is associated with failure to down-regulate the replication initiation proteins. In contrast to normal cervical epithelium the expression levels of Mcm2, Ki67 and geminin expression as expected, are indicative of a hyper-proliferative state.

The mechanisms involved in radiation-induced injury to the bowel and the responses that occur after radiation following normal tissue injury suggest that following mucosal damage, tissue with normal repair capacity may well behave as if in a hyper-proliferative state in response to crypt cell death. This lead me to further questions on the possible role of cell-cycle markers in defining the proliferative state and thus repair capacity of bowel mucosal crypt cells following radiotherapy.

Part III Can Cell-Cycle Markers Predict Symptom Presentation and Severity Of Radiation-induced Bowel Injury (RIBI) after Treatment For Cervical And Endometrial Cancers?

Chapter Five Cell Cycle Phase Specific Markers as Predictive and Prognostic Markers In Radiation-induced Bowel Injury (RIBI) after Treatment For Cervical And Endometrial Cancers?

5.1 Introduction

The prevalence of pelvic radiation disease and significant effect on the quality of life of survivors of cancer demands that future research must focus on understanding the physiological and pathological responses of the intestine to radiation injury. It remains unclear why some women will develop symptoms of radiation-induced bowel injury (RIBI) and others do not. It has also been difficult to predict which women will experience chronic mild to moderate symptoms that either resolve over time, or persist at a manageable level, and those as risk of severe symptoms affecting their quality of life, which often progress or remain unresolved even after surgical intervention. As part of investigations for symptoms of RIBI, an endoscopic assessment and diagnostic biopsy of areas of abnormality or 'inflammation' has become part of the work-up for patients in centers that manage gastrointestinal symptoms after radiation therapy.

It is imperative that future research studies focus on finding predictive methods to identify patients with a high risk of developing healthy tissue toxicity. Ongoing molecular epidemiology research aimed at identifying genetic or epigenetic characteristics that confers a susceptibility to delayed radiation-induced bowel injury must become a priority (Hauer-Jensen et al, 2014).

In this chapter I have attempted, using archived colonic biopsy samples from patients who underwent endoscopic assessment at symptom presentation, to investigate whether variations exist in the cell-cycle status of the proliferation compartment in colonic crypts, and to correlate the information obtained to variations in repair capacity of the proliferating cells in the intestinal villi, severity of symptoms of RIBI and other variables.

5.1.1 Cell Organisation in the Colonic Crypt of the Intestinal Epithelium

The bowel mucosa is a layer of rapidly self-renewing epithelial cells, responsible for absorption of nutrients and water but also acts as a protective barrier against pathogenic microbes. The functional cells of the mucosal surface of the gastrointestinal epithelium

maintain haemostasis by mechanisms that regulate the shedding and replacement of functional cells in the intestinal villi. New functioning epithelial cells are produced from proliferating stem cells and their progeny. These stem cells are found within millions of crypts spaced along the length of the gastrointestinal tract within the millions of villi. New cells are produced in the proliferative compartment of these crypts and each survives through out the lifetime of an animal by regulating the balance between programmed cell death (apoptosis) and renewal (Li et al, 1994). The progenitor cells responsible for replacement and renewal are located in the crypt base (Potten et al, 1990). As daughter stems cells are produced from cell division, the cells move higher up along the crypt and divide, differentiate and mature, giving the differentiated middle compartment of the crypt.

Experimental studies (Potten and Hendry 1983; Potten et al, 1990; Potten and Booth 1997) have been able to determine the location or exact position of stems cells along the crypt-villus axis (Figure 5.1). In the small intestine, the stem cells are thought to reside at positions 4-5 (four or five cells up from the crypt base). In the normal colon, the stem cells are found at the crypt base (positions 1-2). New functioning epithelial cells are produced from these proliferating stem cells and their progeny.

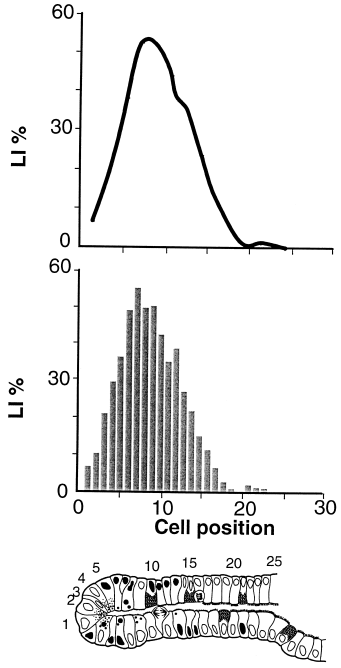


Figure 5.1. Cell position in the crypt base and positional frequency of markers (titrated thymidine in this example) (LI%), recorded in intestinal crypts – (Potten and Booth, 1997). Positive cells after staining can be counted in many sections to calculate the frequency of an event at each cellular level position.

The entire intestinal epithelium is replaced every 2-3 days in mice and every 3-5 days in humans. This requires tight regulation of cell production, differentiation, migration and turnover. Malfunctions in the cell turnover regulation have been linked to inflammatory bowel disease, formation of adenomas and eventually malignant tumours (van der Wath et al, 2013).

The mechanisms controlling this regulation and the self-renewal machinery by cell division and differentiation to replenish lost functional cells and repair tissue following injury are not completely well understood. There is evidence that suggests this tightly controlled mechanism includes some asymmetric chromosome segregation in stem cells and the re-activation of dormant stems cells following injury (Wilson et al, 2008). Crypt cells have the ability to detect induced damage and either ignore, repair or delete cells from the system by apoptosis (Potten and Booth, 1997).

Labelling index studies (de Rodriguez et al 1979; Potten et al, 1982) have provided information about the details on distribution of proliferative cells along the length of the colonic crypt. Labelling index (LI) in the crypt is a measure of mitotic activity along the crypt and is defined as the number of cells in the S phase of the cell-cycle at the vertical position divided by the total number of cells in that position. These studies have shown a distinct ordering of proliferative cells at the base of the crypt (B) and mature differentiated cells at the superficial end (S) opening into the lumen.

In recent years, Ki-67 immunostaining (Batlle et al, 2002; Hanna-Morris et al, 2008; Leedham et al, 2012) has been used as a marker of cell proliferation and has shown a distinct boundary between the proliferative cells and mature cells, with proliferative cells primarily occupying the lower third/ base (B) of the crypt. Recent biological research focusing on finding markers for intestinal stem cells within the proliferative compartment of the crypt has provided more evidence to suggest the direct progeny of proliferative stem cells proliferate rapidly and migrate along the crypt wall towards the superficial third (Potten et al, 1990; Potten et al, 2009).

5.2 Effect of radiation on cell proliferation kinetics

The balance against the probability of achieving local tumour control and the damage induced by radiation is dependent on the proliferation kinetics of dose-limiting normal epithelial tissue. The rate of development of radiation damage is intimately linked to their cell population kinetics. The interval between fractions in a therapeutic regime is generally thought to give time for repair of (reversible) damage, giving time for re-population of 'normal' epithelial cells. Studies developed to determine the response of normal tissues to fractionated radiation, have assumed that the target stem cells have similar radiation sensitivity throughout the regime (Morris et al, 1996).

5.2.1 Radiation sensitivity and the cell cycle

The variation in observed radio-sensitivity of cells in different phases of the cell cycle was first published in the 60s (Terasima et al 1963; Sinclair et al 1966). Cells in mitosis (M) and late G2 phases of the cell cycle are most sensitive to radiation, whilst cells in late S phase are most resistant. Cells in early S phase and G1 would usually demonstrate intermediate radiosensitivity (Withers, 1975). One of the first effects of ionizing radiation on a proliferative cell population is the induction of a delay in cell cycle progression. This delay is temporary and arrests the progression of the cell cycle through G1, S or G2 (Maity et al, 1994) and invariably induces an accumulation of cells at G1/S and G2/M interphases resulting in a synchronized progression of cells into the first post-irradiation cell division.

The duration of the mitotic delay is directly related to the radiation dose and the cell doubling time of the proliferation compartment within the epithelial tissue. The doubling time is defined as the time taken for the renewal of all the epithelial cells in the proliferative compartment. Due to the presence of non-proliferative cells in the proliferative compartment, the doubling time is invariably longer than the cell cycle time.

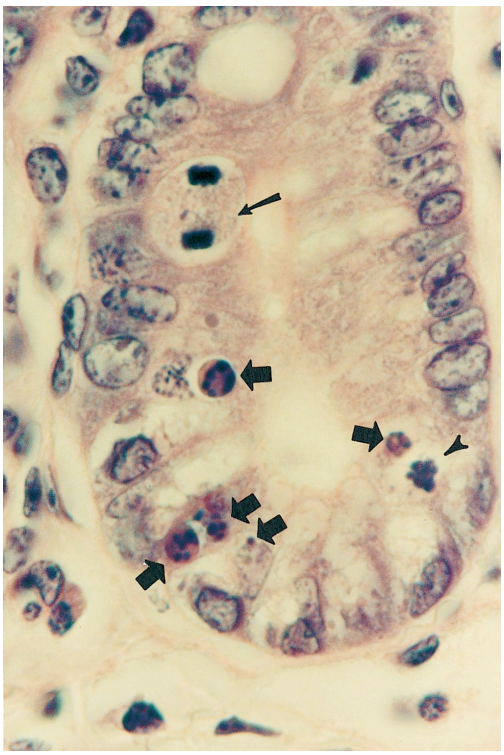


Fig 5.2. A typical longitudinal section from a small intestinal crypt from a mouse 4.5hr after 1 Gy dose of radiation. (Potten and Booth 1997). Cells in the mitosis can be seen (arrow: anaphase, arrowhead: metaphase). Both mitosis and apopotosis involve chromatin condensation and can be difficult to distinguish. Haemoatoxylin and eosin stained (x500)

Mitotic delay in the intestinal epithelium depends on the position of cells in the crypt. A single dose of 1Gy results in a delay in cell cycle progression which approximates to 13% of the doubling time, at cell positions 1-5 at the base of the crypt and falls to ~8% of the doubling time at cell position 6 and upwards (Leschert 1967; Chwalinski and Potten 1986; Potten 1990).

After exposure to 1Gy of irradiation, there is a progressive dose-dependent decline in crypt cellularity, demonstrated in mice small intestine that leads to denudation of the surface mucosa within days after irradiation (Potten 1981,1990; Potten et al 1975, 1990). Cells in the epithelium continue to proliferate during the initial recovery from radiation

exposure, however some cells degenerate and undergo apoptosis and necrosis. Over the course of fractionated radiation, the number of cells that degenerate increases, with no clear evidence that this is dose-dependent (Devik, 1971). Repopulation after irradiation is determined by the cell population characteristics and kinetics of the normal non-irradiated epithelium.

In rapidly proliferating epithelium of the gastrointestinal tract, this occurs shortly after irradiation. The time of onset of this repopulation of cells lengthen during a course as the turnover of cells in the basal layer of the crypts increases progressively. The recovery of these cells from the mitotic delay with an increase in cells undergoing mitosis, demonstrated by an increase in cell labeling indices to 'normal' control levels, marks the beginning of repopulation. Figure 5.2 demonstrates the staining of crypt basal cells after radiation exposure. In the acute phase of recovery, the timing of repopulation appears to be determined by radiation dose, the overall recovery of cells and subsequent repair of tissue injury appears to be related to other intrinsic factors, as inter-patient variability in tissue repair would suggest. It is recognized that cells exhibit a variability in radio-sensitivity with respect to their position in the cell cycle at the time of exposure to radiation, suggesting some normal tissue cells' proliferation kinetics are unaltered by exposure to radiation (Morris GM, 1993). The evidence from experimental studies investigating the variations in radio-sensitivity and the effects of radiation exposure on cell cycle progression suggests that variations exist in this initial tissue injury response (Gilbert et al, 1965).

5.2.2 Radiation and Cell-cycle dynamics in the Gastrointestinal Tract

It is widely accepted that radiation predominantly kills rapidly proliferating cells such as progenitor cells in the intestinal crypts, which leads to insufficient replacement of the villus epithelium (Potten, 1977). It remains unclear how the responses to radiation in the acute phase and the repair capacity of individual patients is related to the proliferation status at the end of a course of radiotherapy.

Whilst some patients present with chronic symptoms associated with the TGF-B mediated fibrosis in strictures and new vessel formation and telangiectasia, others will present with symptoms of chronic insufficiency of the functional cells of intestinal mucosa resulting from loss of stem cells, incomplete healing and ischaemia (Figure 5.3).

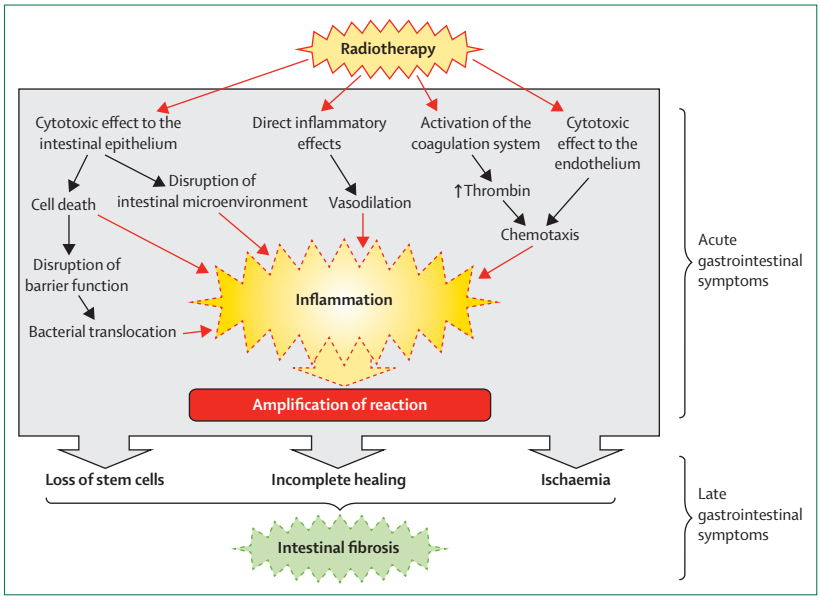


Figure 5.3. Mechanisms of radiation-induced bowel injury (Ferreira et al, 2014). Several mechanisms act concurrently to provoke gastrointestinal symptoms after radiation.

5.3 Radiation-Induced Bowel Injury as a model of Inflammatory Bowel Disease (IBD)

Animal models describing the pathology and patho-physiology of Inflammatory Bowel Disease (IBD) have confirmed in patients models, the identical patho-physiology in RIBI (Hauer-Jensen et al, 2014). Whilst in inflammatory bowel disease, the 'toxic' agent, or 'insult' has yet to be clearly identified, dose-response animal models in radiation make it possible to continue to investigate relationships between radiation exposure and normal tissue responses (Hauer-Jensen et al, 1988), however it remains difficult to extrapolate

results from animal models to the understanding of radio-sensitivity, repair capacity, and inter-patient variation in responses to treatments.

Research into IBD is overwhelmingly more prevalent that studies related to RIBI. The prevalence of pelvic radiation disease remains higher and continues to rises. There are no reliable markers currently in widespread use in clinical practice that give an indication of disease activity and degree of mucosal inflammation in the bowel to enable monitoring of IBD disease activity. In an experimental study using sections from colonic biopsy and resection specimens of 48 patients with IBD, Davies et al, (2003) investigated 5 patients inactive/quiescent Crohn's disease (CD), 13 with active CD, 19 with inactive/quiescent Ulcerative Colitis (UC), 11 with active UC, and 15 normal controls. Sections were immunostained with antibodies to Mcmc2 and Ki-67. Labelling index (LI) was determined by calculating the percentage of immunopositive epithelial nuclei for the entire glands, and for gland thirds (superficial, middle and basal). Mcm2 LI was increased in the superficial third in active vs inactive/quiescent UC and Crohn's. The Mcm2 Li was significantly greater than Ki67 in active IBD both in entire glands and in gland thirds. Mcm2 LI for entire glands were significantly higher in UC (all cases) compared to CD. This study showed an increase in cell cycle entry as indicated by expression of Mcm2 (and to a lesser extent Ki67) in the superficial third of colonic glands in active disease compared to inactive disease. Figure 5.4 shows staining in normal and IBD colon from the study.

This increased expression in superficial third of the glands suggests an on-going subclinical microscopic chronic inflammation in the epithelial cells at the mucosal/luminal interface and raised interesting questions about the role of cell-cycle markers adding immunohistochemical support to the histopathological diagnostic features which to this day, fail to identify patients at increased risk of ongoing inflammation and carcinogenesis. Cell-cycle entry of epithelial cells in the middle and superficial thirds of glands in active IBD has been shown to be consistent with the local release of pro-inflammatory cytokines in active inflammation, together with a direct response to injury of epithelial cells (Rhodes and Campbell, 2002).

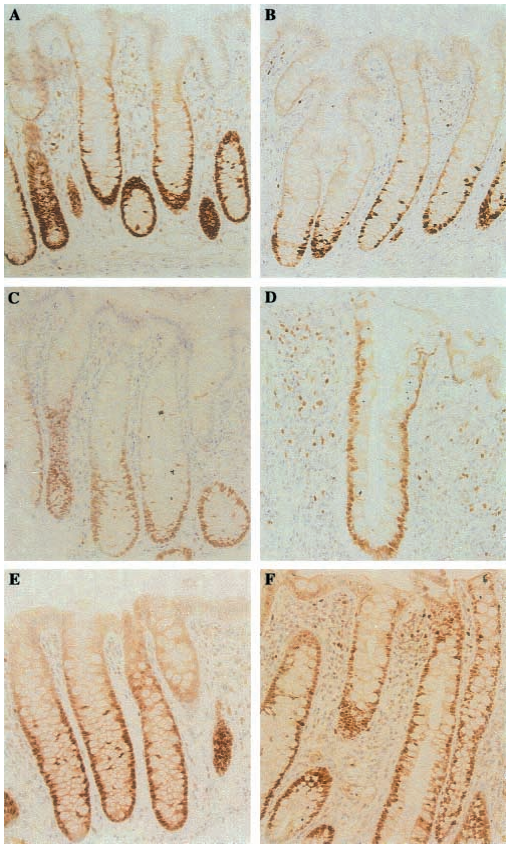


Figure 5.4. Immunostains showing Mcm2 and Ki67 staining in normal colon, active and inactive IBD (Davies et al 2003). (A) Mcm2 and (B) Ki67 expression in normal colon. Both proteins are confined to the basal third of the glands, with Mcm2 being expressed more frequently than Ki67. ((C) quiescent CD, (D) active CD, (E) quiescent UC and (F) active UC. Cells expressing Mcm2 are present in the superficial third of the glands in regions of active inflammation, but are absent from this location in inactive/quiescent disease

Other studies (Bortoluzzi et al, 1995; Gloria et al, 1996) have investigated proliferative activity in colonic mucosa in IBD patients as a marker of ongoing inflammation.

5.4 Cell-Cycle markers in the Intestinal Mucosa

The use of cell-cycle markers in clinical oncology and multiparameter analysis of Mcm2, geminin and Ki-67 was described in Chapter 4. In this section, I will attempt to describe their use in assessing proliferative status in colonic (rectal) crypts in normal and radiation-exposed samples. The loss of proliferative capacity and cell-cycle withdrawal following engagement of the somatic differentiation program is tightly coupled to down-regulation of core constituents of the DNA replication licensing machinery, including the Mcm2-7 proteins. This coupling between loss of proliferative capacity, cell cycle withdrawal, down-regulation of the Mcm2-7 helicase complex and differentiation has been observed in anal, bladder, cervical, oesophageal, oral, pancreatic, prostate and also colonic epithelia (Kayes et al, 2009)

Hanna-Morris et al (2009) investigated the sensitivity of Mcm2 over Ki-67 as markers of mucosal crypt cell dynamics. 'Normal control' archived samples were obtained from patients undergoing colorectal resection for benign conditions who had no history of colorectal cancer or inflammatory bowel disease. Mcm2 staining suggested that significantly more normal control mucosal cells were proliferating in all crypt compartments than was demonstrated by Ki67 staining. The difference was larger for the middle and superficial compartments (Figure 5.5). Minichromosome maintenance (MCM) proteins are demonstrated in early G1 phase when Ki-67 cannot be detected. They are essential for replication and hence loss of cell cycle control is associated with increased MCM protein expression. In this study I will attempt to define the proliferation status of the radiation-exposed colonic crypt compartments in patients investigated for symptoms of RIBI and its clinical relevance.

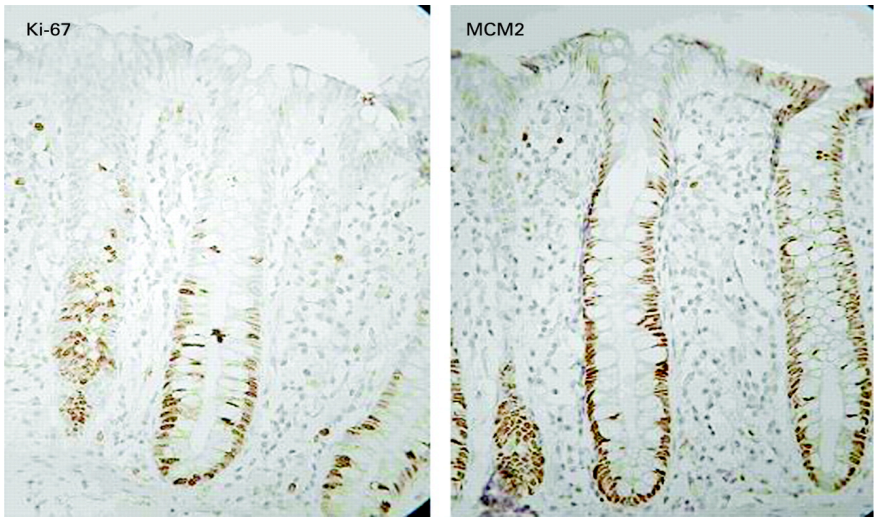


Figure 5.5. Sections of macroscopically normal mucosa sampled 10cm proximal to colorectal adenocarcinoma immunostained for Ki-67 and Mcm2 (Hanna-Morris et al, 2009). A higher proportion of nuclei in the superficial and middle compartments staining for Mcm2 expression is demonstrated (magnification X 250)

5.5 Materials and Methods

5.5.1 Study Cohort

Sections of archival formalin-fixed, paraffin-embedded from 72 rectal biopsy samples were obtained from diagnostic biopsy specimens. Normal colo-rectal tissue samples were used as controls (n=16), whilst 56 samples were from patients who presented at oncology follow-up with symptoms of radiation-induced bowel injury (RIBI) after treatment for cervical and endometrial cancer. Patients were identified from the UCLH Oncology and Gastroenterology database. Ethics approval was obtained from the local research ethics committee; University College London/University College London Hospitals Committees on Ethics of Human Research. All samples were anonymised using Freezerworks in accordance with the UCL/UCLH Biobank.

Clinical data was extracted from hospital records on patient and cancer demographics, oncology follow-up, type and dose of radiotherapy received, chemotherapy received, date

of presentation with bowel symptoms, nature of symptoms at presentation to oncologist and gastroenterologist, histological features reported from biopsy samples, and status of RIBI-related symptoms at last follow-up. The normal colo-rectal samples were obtained from endoscopic biopsy samples from female patients without a diagnosis of cancer or inflammatory bowel disease who underwent investigations for unrelated symptoms and who had never received any radiation treatment. As all sections were anonymised, clinical data was blinded until data analysis was performed.

5.5.2 Antibodies.

The primary antibodies used were mouse monocloncal Mcm2 antibody (clone 46) obtained from BD Transduction Laboratories (Lexington, KY, USA). Ki-67 monoclonal antibody (Mab) (clone MIB-1) was obtained from DAKO (Glostrup, Denmark) Rabbit poloclonal antibody against human geminin were previously generated and validated at the UCL laboratory (Leica Biosystems, Cat No. NCL-L Geminin).

5.5.3 Immunohistochemistry

Sections were cut from each paraffin-embedded tissue block of colo-rectal tissue. Three-micrometer sections were cut onto Superfrost Plus slides (Leica Microsystems), dewaxed in xylene, and rehydrated through graded alcohol to water. For antigen retrieval, slides were pressure cooked in 0.1ml citrate buffer (ph 6.0) at 103kPa for 2.5min. Tissue sections were immunostained using the Bond Polymer Define Detection Kit and Bond Polymer Define Detection kit and Bond-X automated system (Leica) according to the manufacturer's instructions. Primary antibodies were applied at the following dilutions: Ki67 (MIB-1); 1:70, Mcm2; 1:2000 and geminin (1:200) and incubated for 49 minutes. Coverslips were applied using Pertex mounting medium (Cellpath).

All antibodies were diluted using Bond antibody diluent. Antigen retrieval protocol as follows - Mcm2; 30mins ER1 (EDTA-based); ph 6.0 at 100degrees centigrade, Geminin; 30 mins ER2 (EDTA-based), ph 9.0 at 100 degrees centigrade. Endogenous peroxidase was blocked using 3-4% (v/v) hydrogen peroxide for 5 mins prior to the primary antibody application. Haematoxylin applied for 2 mins to counterstain; both included as part of the Bond Polymer Refine Detection kit. For all tissues, incubation without the primary antibody was used as a negative control and tonsil epithelium was stained for marker expression as positive control.

5.5.4 Quantification of staining results

A quantitative value for the degree of expression of Mcm2, Ki-67 and Geminin was obtained by calculating a labeling index (LI) for each marker, representing the percentage of epithelial nuclei that were immunopositive. Three to five crypts were assessed for each case for each marker. LIs were calculated by counting both the iummunopositive cells in the entire glands and gland thirds and cells that did not stain for the markers. The LI percentage was calculated as (number of positive cells/total number of cells x100. The gland thirds represented the superficial (S), middle (M) and basal (B) thirds and were defined by measuring the crypt length and dividing by three. Figure 5.6 below demonstrates this in one of my stained slides. For all sections the slides were counted individually by myself and confirmed by 2 research assistants also blinded to clinical data (SD, CI). Median and mean LIs were determined for entire crypts and for superficial, middle and basal compartments.

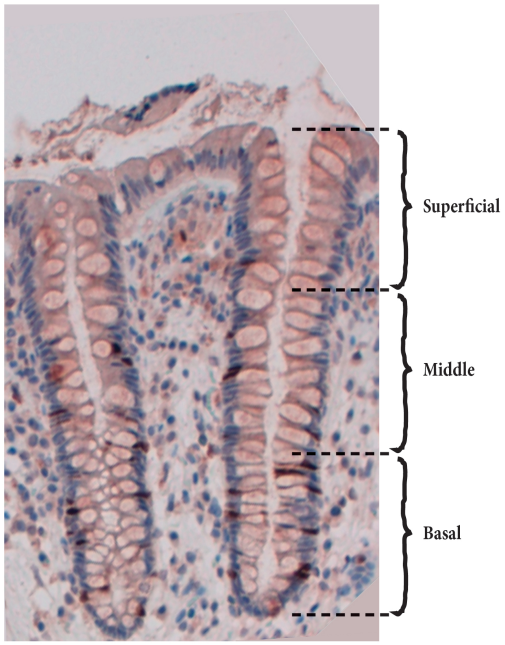


Figure 5.6 Hamatoxylin and eosin stained section of normal colon, demonstrating the division of a colonic crypt into superficial, middle and basal thirds for calculation of LIs

5.5.5 Statistical Analysis

Mcm2, Ki-67 and Geminin labeling indices (LIs) for entire glands and gland thirds were compared using Kruskal-Wallis test. Differences in LIs for Mcm2, Geminin and Ki-67 groups were assessed using the Wilcoxon signed rank test. To compare normal colon and RIBI marker espression, Mann-Whitney test was used

The aim of the study was to explore differences in both the proliferative compartment (basal third), and determine evidence of increased cell cycle entry in the superficial third of the glands in patients who had symptoms of severe disease. I also sought to compare differences in in relation to findings on histopathological assessment. Crypt cell dynamics where compared for the 3 previously defined presenting symptom 'clusters' using Mann-Whitney Test. Pearson correlation and mean ranks were calculated to assess

for a relationship between crypt third marker expression and interval to presentation. All analyses were carried out using SPSS version 21 - IBM (SPSS Inc., Chicago, USA).

5.6 Results

5.6.1 Cell-cycle marker expression in Normal colonic crypts (controls)

Expression of Mcm2, Ki-67 and Geminin in normal colon was largely confined to the basal third of the glands as previously reported (Freeman et al, 1999; Davies et al, 2002). There was a statistically significant difference found when comparing expression of markers between gland thirds Mcm2, Ki-67 and Geminin ($P<0.0001$). Mcm2 LI was significantly greater than the Ki-67 LI for entire glands and for the basal thirds ($P<0.0001$). Table 5.1 shows the percentage LI for crypt compartments for all three markers stained, with evidence of a predominance of proliferating cells in the basal third. Figure 5.7 shows results demonstrated in box-and-whisker plots.

Marker	Crypt Thirds	Median (Range)%	p values
Mcm2	Superficial	7.5 (0 – 44)	
	Middle	55.4 (4 – 85)	
	Basal	83.8 (51.7 – 91.5)	
Ki-67 (MIB-1)	Superficial	5.1 (0 - 20)	
	Middle	34.3 (3.2 – 67.7)	<0.0001*
	Basal	66 (39 – 84)	
Geminin	Superficial	0 (0 – 3)	
	Middle	8.6 (2.6 – 20.4)	
	Basal	21.1 (9.5 – 37)	

Table 5.1. Cell-cycle marker expression in normal colo-rectal controls. Results confirm the prescence of cycling cells predominantly in the basal compartment, with high expression of Mcm2, Ki-67 and Geminin expression.

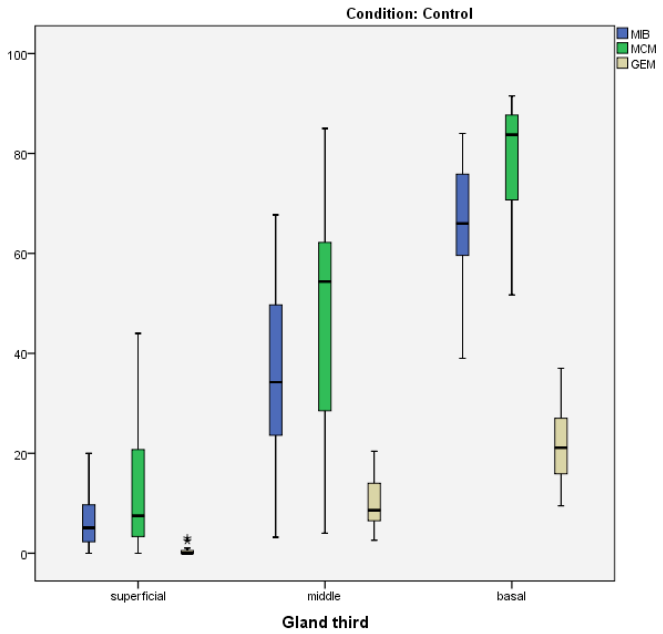


Figure 5.7. Box and Whisker plots demonstrating marker LIs for Normal Colon.

5.6.2 Cell-cycle marker expression after radiotherapy in patients who presented with symptoms of RIBI

In patients who presented with symptoms of radiation-induced bowel injury (RIBI), rectal biopsy samples at gastroenterology assessment were immunostained to assess expression of Mcm2, Ki-67 and Geminin. There was a statistically significant difference in cell-cycle marker expression between all compartments ($P<0.0001$). Expression of all 3 markers was mainly confined to the basal third of the crypt.

The median values and range of LI percentages are shown in Table 5.2. Figure 5.8 illustrates the results in a box-and whisker plot showing Li for all markers in the crypt thirds. Although median Ki-67 was greater than Mcm2 in the group, this was not statistically significant (Wilcoxon test; $p = 0.08$).

Marker	Crypt Thirds	Median (Range)	
			p values
Mcm2	Superficial	0.7 (0 -27.0)	
	Middle	5.0 (0-55.7)	
	Basal	18.3 (0-97.0)	
Ki-67 (MIB-1)	Superficial	1.0 (0 – 33.5)	
	Middle	10.3 (0 – 76.0)	<0.0001*
	Basal	27.6 (0- 82.0)	
Geminin	Superficial	0 (0-20.1)	
	Middle	6.2 (0- 31.0)	
	Basal	11.6 (0 – 41.2)	

Table 5.2 Cell-cycle marker expressions in colorectal mucosa of patients who presented with symptoms of RIBI. LI for all markers was significantly greater in the basal third.

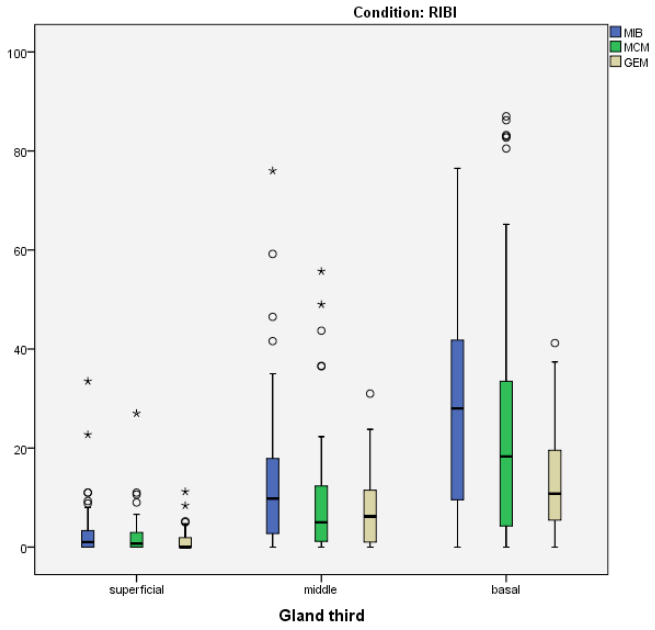


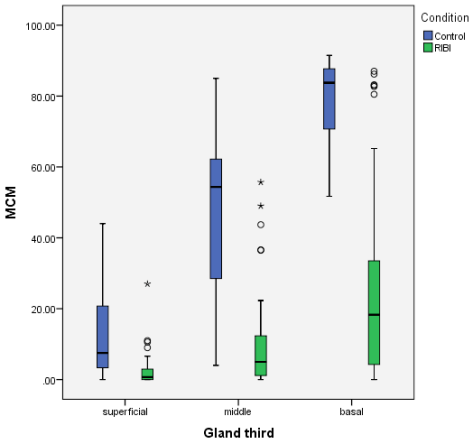
Figure 5.8. Box and Whisker plots demonstrating LIs in patients who presented with symptoms of RIBI

5.6.3 Comparing Cell-cycle marker expression and proliferative states in normal colon and RIBI

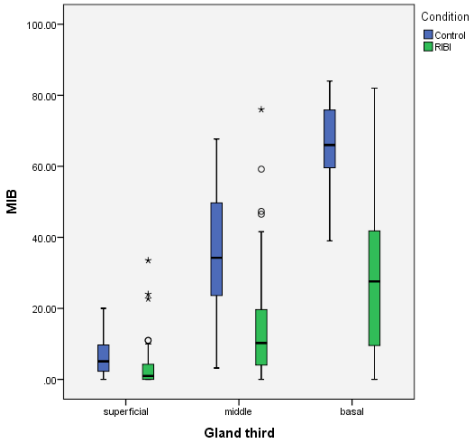
In patients who presented with symptoms of RIBI compared to normal colon, there was a statistically significant difference in Mcm2 LI and Ki-67 LI for entire crypts and all crypt thirds ($p<0.0001$), with a significantly lower expression of markers of proliferation in the basal third. Geminin LI was significantly lower in RIBI samples compared to normal colon for the basal third ($p=0.004$) and for middle thirds ($p=0.026$) although median LIs were 6.2% and 8.6%. Table 5.3 shows results of the comparison of RIBI samples compared to normal colon. A box-and-whisker representation of all markers in both groups is shown in Figure 5.9. Figure 5.10 shows nuclear staining in all compartments for cell-cycle markers investigated in both normal controls and in a sample from a patient who presented with symptoms of RIBI.

Marker	**Crypt Thirds**	**Normal Control** Median (Range)	**RIBI** Median (Range)	**p- values ***
Mcm2	Superficial	7.5 (0 – 44.0)	0.7 (0 -27.0)	<0.0001
	Middle	55.4 (4 – 85.0)	5 (0-55.7)	<0.0001
	Basal	83.8 (51.7 – 91.5)	18.3 (0-97.0)	<0.0001
Ki-67 (MIB-1)	Superficial	5.1 (0 – 20.0)	1 (0 – 33.5)	0.009
	Middle	34.3 (3.2 – 67.7)	10.3 (0 – 76.0)	<0.0001
	Basal	66 (39 – 84.0)	27.6 (0- 82.0)	<0.0001
Geminin	Superficial	0 (0 – 3)	0 (0-20.1)	0.401
	Middle	8.6 (2.6 – 20.4)	6.2 (0- 31.0)	0.026
	Basal	21.1 (9.5 – 37.0)	11.6 (0 – 41.2)	0.004

Table 5.3. Mcm2 and Ki-67 LIs in entire crypt and all crypt thirds were significantly lower in RIBI samples compared to normal control. Median LI for Geminin for both groups was 0%. Geminin LI was significantly lower post-radiation in patients presenting with RIBI. * Mann-Whitney Test



(A)



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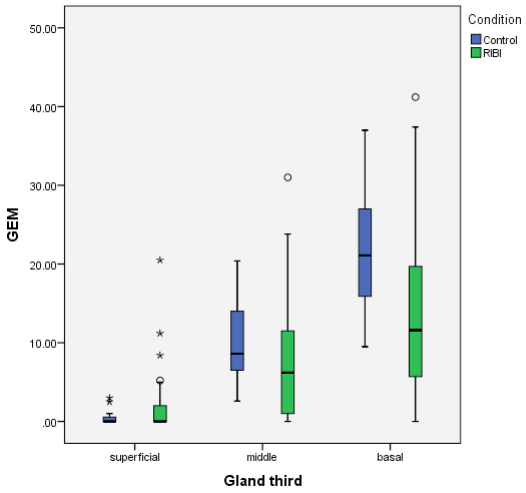


(C)

Figure 5.9. Box and Whisker plots comparing normal colon controls and RIBI marker expression in (A) Mcm2 (B) Ki-67 (C) Geminin

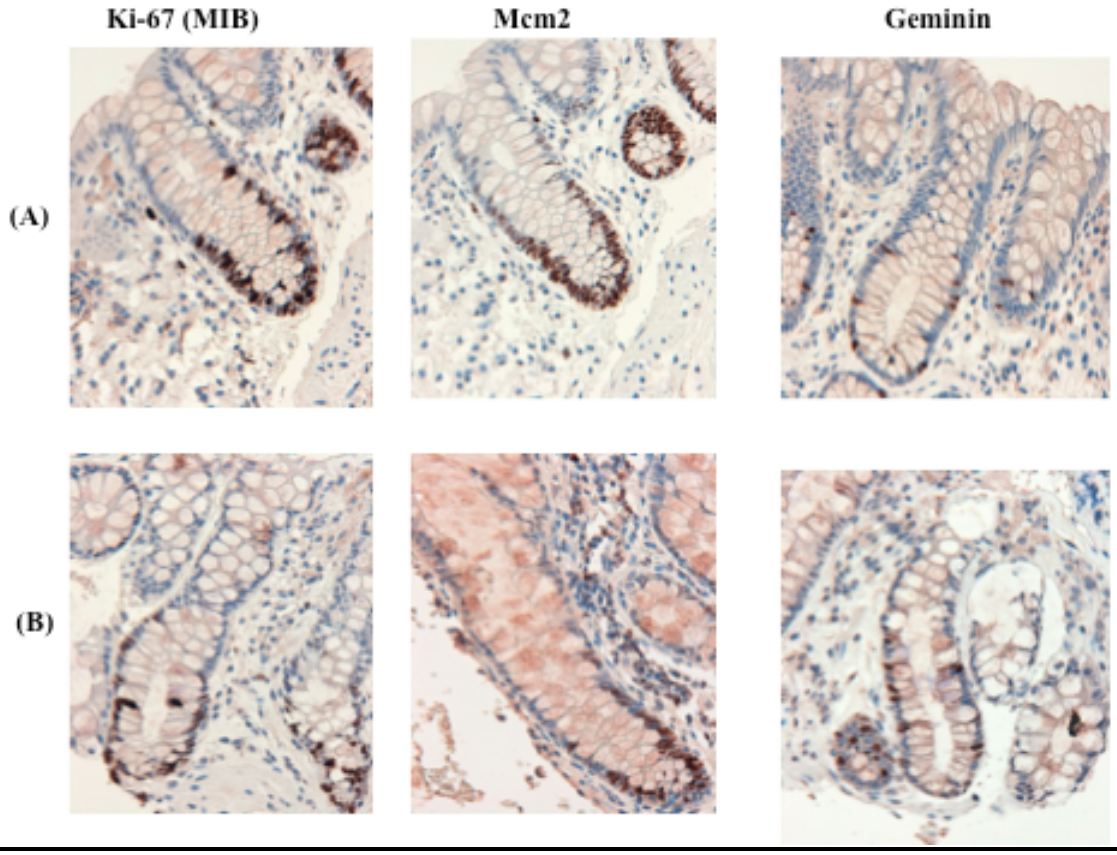


Figure 5.10. Sections of (A) normal mucosa and (B) bowel mucosa from patient with symptoms of radiation-induced bowel injury (RIBI) (magnification X 200). Immunostaining demonstrated predominantly in the lower thirds of the crypts. This patient presented at 6 months after completion of radiotherapy with diarrhoea, increased frequency, urgency and mild urge incontinence. Endoscopic findings reported mild oedema and congestion from the anal verge to descending colon with some telangiectasia. Biopsy findings were normal. The patient still had ongoing mild sympotoms at the time of analysis despite intervention.

5.6.4 Relationship between Cell-cycle marker expression and interval to presentation with RIBI

The median interval from completion of radiotherapy to presentation was 8 months (range 4 – 50). Samples were taken at gastroenterology assessment at the time of presentation with symptoms of RIBI. Pearson correlation showed a statistically significant correlation between Mcm2 expression in the superficial (p=0.008) and middle (0.005) thirds only. There was a trend towards significance for Mcm2 expression in the basal third of the crypts (p=0.084). The longer the interval to presentation, the higher the frequency of Mcm2 expression. This difference was not found for ki-67 expression. Table 5.4 shows correlation coefficients for marker LIs for gland thirds.

	MIB			**MCM**			**GEM**		
	S	**M**	**B**	**S**	**M**	**B**	**S**	**M**	**B**
INTERVAL TO PRESENTATION*	0.14	0.188	0.074	**.436**	**.462**	0.292	0	0.117	0.044

Table 5.4. Correlation between marker expression and interval to presentation in patients presenting with RIBI. Values represent the pearson correlation coefficient for each marker for all crypt thirds. Statistical significance was found for Mcm2 only; superficial and middle thirds.

5.6.5 Relationship between Cell-cycle marker expression and severity of symptoms of RIBI

There was no statistical difference found for Mcm2, Ki-67 and Geminin Lis for crypt thirds in patients who had complete resolution of symptoms compared to those with ongoing mild/moderate symptoms, and those with severe symptoms of RIBI at the time of analysis. Table 5.5 below shows results. Figure 5.11 shows marker expression in crypt thirds for each marker for status of RIBI symptoms

		RIBI – Mean Ranks for marker LIs			
		Symptoms Resolved	**Mild/Moderate**	**Severe**	**p-value***
Mcm2	S	18.16	18.13	17.43	0.260
	M	19.00	18.13	15.50	0.675
	B	17.50	19.58	16.43	0.896
Ki-67 (MIB-1)	S	16.62	22.47	22.93	0.986
	M	19.03	22.00	18.07	0.752
	B	19.41	19.83	21.79	0.783
Geminin	S	23.88	17.07	16.86	0.126
	M	20.59	20.60	17.29	0.785
	B	20.38	22.37	14.00	0.272

Table 5.5 Table showing Mean Rank scores for marker LI showing no relationship to RIBI symptoms status at last follow-up. Mann-Whitney test (non-parametric) for (n=39 with missing data). There was no statistical difference found for crypt thirds, although not statistically significant, the mean rank scores were lower in the basal third/proliferative compartment for patients with ongoing severe symptoms of radiation-induced bowel injury at time of analysis.

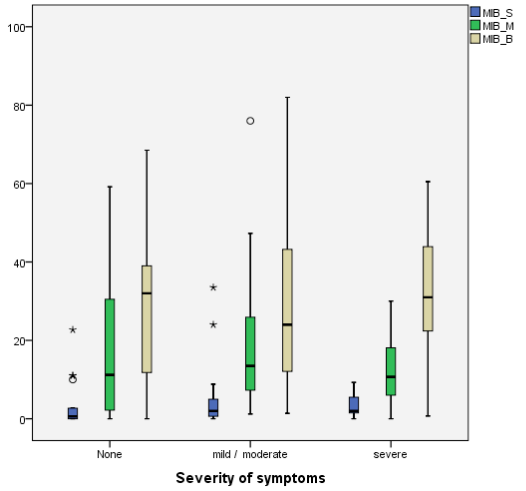
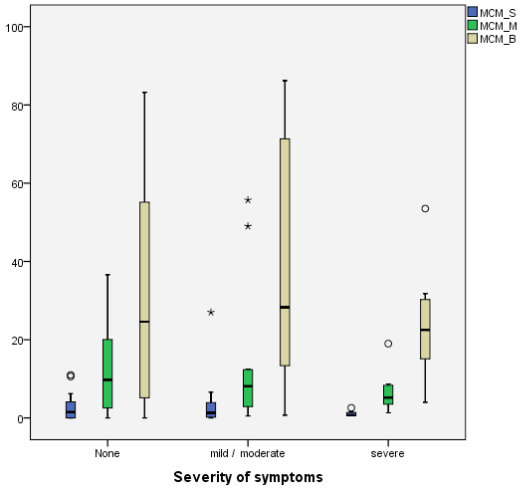


Figure 5.11(A)



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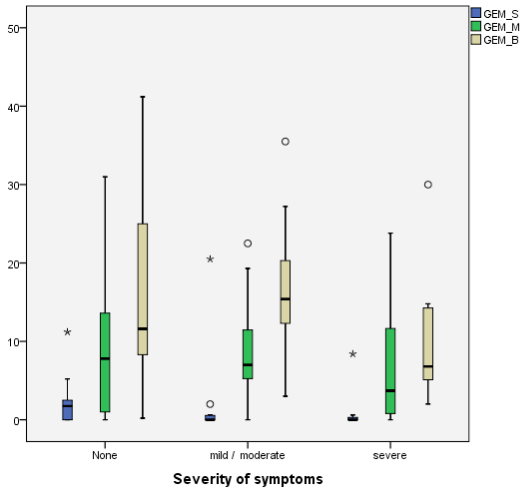

(C)

Figure 5.11. Box and Whisker plot showing marker expression in patients presenting with RIBI by severity of symptoms (A) Ki-67 (MIB) (B) Mcm2 (C) Geminin. Patients with resolved symptoms (None); n=17. Mild/moderate – mild symptoms not affecting quality of life; n=14, managed with dietary manipulation and/or Imodium/codeine; moderate symptoms requiring further investigations; n=1. Severe symptoms requiring surgery and/or TPN even after surgical intervention; n=7.

5.6.6 Relationship between Cell-cycle marker expression and presenting symptom cluster

Factor analysis in chapter 2 was used to define 3 presenting symptom factors or 'clusters'. There was a statistically significant difference in expression of both Mcm2 (p= 0.022) and Ki-67 (MIB) (p=0.032) for the basal third for patients presenting with symptom cluster/factor score 2 (diarrhoea, loose stools and increased bowel frequency – bowels open (BO) >4times/day) and faecal incontinence) only. No statistically significant correlation was found for factor scores for presenting symptoms cluster and crypt marker expression for Geminin. Table 5.6 shows Pearson correlation coefficients for crypt thirds for all markers in the 3 symptom clusters.

	MIB_S	MIB_M	MIB_B	MCM_S	MCM_M	MCM_B	GEM_S	GEM_M	GEM_B
Factor 1	-0.046	0.072	0.124	-0.058	-0.199	-0.236	-0.084	-0.165	-0.032
Factor 2	0.049	0.111	**0.352**	0.191	0.248	**0.348**	-0.235	-0.144	-0.071
Factor 3	-0.013	0.074	0.134	0.122	0.012	-0.024	-0.074	-0.186	-0.032

Table 5.6. Correlations of Factor Analysis (Symptom Presentation Cluster) Scores with Crypt third Marker Expression. Values represent the Pearson Correlation coefficients between factor scores and expression of markers. Increased Ki67 (MIB) and MCM expression was found in the basal zone with increased scoring for factor/cluster 2 only. Factor/Cluster 1- nausea, vomiting, symptoms and signs of acute or subacute bowel obstruction; Factor/Cluster 2- increased bowel frequency, loose stool, diarrhoea, faecal incontinence; Factor/Cluster 3 - bloating, flatulence, abdominal pain, urgency, rectal bleeding and per-rectal mucus

5.6.7 Relationship between Cell-cycle marker expression and histopathological findings on biopsy

Histological findings on biopsy in patients presenting with symptoms of RIBI ranged from normal to mild non-specific inflammatory changes, and varying degrees of colitis and crypt architecture distruption as reported on histopathological reports. Of all patients with endoscopic and biopsies at presentation with symptoms of RIBI to gastroenterologists, 15/41(36%) had evidence of pathological changes suggestive of radiation changes while 26 of the samples where reported as normal. There was no significant difference in cell-cycle marker expression for crypt thirds between the normal and pathological groups. Mean rank values and p values are shown in Table 5.7.

		Normal Histology	**Radiation changes***	**p-value**
Mcm2	S	26.41	24.93	0.747
	M	26.80	23.89	0.533
	B	27.26	22.68	0.326
Ki-67 (MIB-1)	S	27.94	30.03	0.666
	M	27.83	30.33	0.611
	B	27.55	31.10	0.471
Geminin	S	31.48	22.07	0.041
	M	29.73	2697	0.580
	B	28.70	29.83	0.821

Table 5.7. Comparison of cell-cycle marker expression with normal vs pathological findings of radiation changes on histology (Radiation changes; Mild to Significant colitis with crypt architecture distortion and/or mild fibrosis of lamina propria) (*Mean Rank Values for marker expression)

5.7 Conclusions and Discussion

To my knowledge, this is the first description of the expression of cell-cycle phase specific markers in colo-rectal tissue samples taken from patients with symptoms of radiation-induced bowel injury (RIBI). The expression of cell-cycle states using immunohistochemistry in radiation-induced bowel injury (RIBI) has not been previously investigated. Hanna-Morris et al (2009) report an increased sensitivity of Mcm2 over Ki-67 as markers of colonic mucosal crypt cell-cycle dynamics in active inflammatory bowel disease.

I demonstrate decreased expression of cell-cycle markers Mcm2, Ki67 (MIB-1) and Geminin in colonic crypts exposed to pelvic radiation after treatment for cervical and endometrial cancers. Compared to normal colon, the colonic crypts in women presenting with bowel symptoms after pelvic radiation showed **decreased** cell cycle entry suggesting a reduced capacity of cells in the basal/proliferative compartment. Exposure of normal bowel mucosa to radiation is associated with variable cellular responses; it is presumed that injury to the rapidly dividing and extremely radio-sensitive mucosal crypt cells is predominantly responsible for acute radiation toxicity whilst chronic radiation toxicity is caused by injury to the less mitotically active and less radiosensitive intestinal cells (Bismar and Sinicrope, 2002).

The observed reduction in expression of Mcm2, Ki67 and Geminin in the basal (proliferation) compartment in RIBI samples compared to normal controls were not found to be significant when comparing marker expression with severity of symptoms. I appreciate the limitations of this explorative study, given the small sample size and the retrospectively collection of clinical data. I found a statistically significant correlation between Mcm2 expression in the superficial and middle compartments and the interval to presentation (median; 8 months). This seems to suggest that the longer the interval from the insult (radiation), the higher the percentage of increased cell-cycle entry in the superficial and middle compartments without a significant increase in proliferation in the basal compartment, suggesting limited evidence of improving repair capacity in the basal compartment with time from radiation.

In chapter 2 I used factor analysis to divide 14 items - symptoms (and signs) into 3 presenting symptom 'clusters'. In this cohort study, patients who presented with presenting symptom cluster/factor 3 (*bloating, flatulence, abdominal pain, urgency, rectal bleeding and per-rectal mucus)* were more likely to have severe symptoms of RIBI compared to cluster/factor 2 (*diarrhoea, loose stools and increased frequency and faecal incontinence)* or cluster/factor 1 (*nausea, vomiting, symptoms/signs suggestive of acute or sub-acute bowel obstruction)*. When looking for associations between cell-cycle marker expression and presenting symptom cluster in the present study, I found a statistically significant correlation between marker expression and symptom cluster/factor 2 only. Patients who scored highest for this cluster of symptoms were more likely to have higher marker expression in the proliferative (basal) compartment of crypts, suggesting a higher proportion of actively proliferating cells compare to cluster 3, and thus suggesting that patients scoring higher for cluster 2 were more likely to improve over time.

In summary, we have demonstrated decreased Mcm2, Ki67 and Geminin expression in the basal third (proliferative compartment) of colonic crypts in patients who had endoscopic biopsies taken during investigation for symptoms of RIBI. Though no definite conclusions can be drawn, our results also suggest a relationship between symptom cluster presentation and proliferation (and repair capacity) that needs to be further investigated.

Chapter Six Summary Discussion

Chapter Six Summary Discussion

6.1 Summary of Hypotheses

A national survey of consultant gastroenterologists in the UK (Henson et al, 2012), confirmed that the reporting of toxicity data amongst UK clinicians is poor, and mostly done by history taking. This is still true and outside of clinical trials, retrospective reviews provide unstructured clinician-reported information. In this work, I initially set out to explore the incidence and nature of presentation of radiation-induced bowel injury in women treated for Cervical and Endometrial cancer in a London cancer centre in a retrospective study.

Following this, I then set out to develop a model for a scoring tool as a proposed way of improving the reporting of new bowel symptoms after radiation treatment. The specific patho-physiology of the radiation-induced bowel injury is not always identified during routine investigations. An effective scoring system may well have the potential to aid in the diagnosis of the underlying patho-physiology and guide clinical management.

In search of surrogate markers of radiation toxicity, I applied (to our knowledge, for the first time in cervical cancer) the use of cell-cycle markers using immuno-staining techniques to explore the potential of Mcm2, Ki67 and Geminin as markers of (chemo)-radio-sensitivity. It can be assumed that a marker of radio-sensitivity and tumour response might have a bearing on radiation toxicity, if we are to presume that both sensitivity to tumour and normal healthy tissue is related.

The immuno-histochemical methods used to analyse cervical tumour samples were then utilised to stain for the same cell-cycle markers in colonic crypt cells in women treated with radiation and who subsequently presented with symptoms of radiation-induced bowel injury (RIBI). Rectal samples from women investigated with endoscopy and biopsy were stained with Mcm2, Ki67 and Geminin, multi-parameter analysis of these markers serves as a marker of proliferative status. I conclude with an explorative

analysis of the link between the proliferation status of the bowel mucosa exposed to radiation with the symptom cluster at presentation, severity of symptoms at follow-up, and other secondary endpoints.

6.2 Summary of Results

6.2.1 Retrospective-Prospective Cohort

A review of records of 541 women treated within the North London Cancer Network between 2003 and 2010 with radiotherapy (with or without chemotherapy) for cervical and endometrial cancer identified 152 (28%) women who reported significant new bowel symptoms after pelvic radiation. As all women presented with multiple symptoms, we analysed the presenting clinical features in this cohort of 152 women and therefore by 'clustering' of 14 reported symptoms and signs documented into 3 clusters, we were able to analyse the data in a more practical context. Median follow-up for all patients was 60 months from end of radiotherapy to last oncology follow-up. Univariate analysis showed increasing age, smoking, extended field radiation and cervical cancer (cf endometrial cancer) treatment, as well as the need for surgical intervention to be significant predictors of ongoing disease at last follow-up. In multivariate analysis, the only significant predictors of severity of bowel symptoms after radiation were; age, cervical cancer and patients presenting with symptom 'cluster 3'- (bloating, flatulence, rectal bleeding and per-rectal mucus).

Fifteen women (19%) in the cervical cancer group had RIBI requiring surgical intervention compared with 5 (6.7%) in the endometrial cancer group. Although no differences were found in multivariate analysis for severity of symptoms with the addition of chemotherapy or extended field boosts, our results suggest in keeping with the

literature, that the treatment of cervical cancer (no prior hysterectomy, concomitant chemo-radiation with weekly Cisplatin and 50.4Gy over 5.5weeks plus intracavity brachytherapy; 15Gy in 2 fractions) confers greater risk to normal healthy bowel than in endometrial cancer treatment (pre-radiation hysterectomy, followed by adjuvant radiotherapy - 45Gy over 4 weeks; patients who receive chemotherapy (FIGO stage III/IV) usually receive 6 cycles of Carboplatin and Paclitaxel prior to radiation).

This retrospective-prospective study (patients who completed radiation after 2010 were followed prospectively) revealed the prevalence and features of pelvic radiation disease (28%) in our cohort, with over 10% of patients suffering severe symptoms requiring surgical resection of injured bowel and strictures. Factor analysis in this study enabled an analytical approach to presenting features of bowel injury. Data was collected from patient records and although clinician-reported, highlights the need for patient-reported toxicity tools, practical for use in the clinical setting.

6.2.2 Proposed Model for RIBI Scoring Tool

Whilst recognising that data collected from records at follow-up consultations regarding toxicity is clinician – reported and unstructured, I sought to create a model for a scoring tool using available data from our cohort. Research studies that require long-term follow-up of patients after treatment are difficult to conduct, as patients do not always continue long-term follow-up within the cancer centre were treatment was received. Andreyev et al, (2013) in the ORBIT trial showed algorithm-based care using a patient- reported structured questionnaire (IBDQ-B), in the clinical trial setting could be administered by a nurse.

Given the increasing pressure on services both in terms of time allocated to clinics and the feasibility of collecting toxicity data with patient-reported questionnaires, which usually have to be completed before consultation, I proposed in this chapter, a template for a scoring tool. The ideal scoring tool is easy to use, patient and clinician reported,

and easily reproducible. Collection of toxicity data should enable identification of the specific sites and nature of bowel injury and analysis of long-term follow-up data should enable greater insight into the patho-physiology of radiation-induced bowel injury.

Data from our cohort study of 152 women was re-visited. Median follow-up time was 60 months. The scoring model was developed by dividing the cohort into 2 sub-sets; a 'test' set (n =75) which was validated on a 'confirmatory' set (n=77). Factor analysis used re-used to 'cluster' 14 presenting symptoms and signs into the 3 groups as defined in chapter 1. Predictive accuracy of the score (for severity of symptoms) was compared by the area under the receiver operating characteristics curve (ROC) for each 'symptom cluster' /'factor' score as well as a total score for the three clusters.

The score proposed (see template in chapter 3) should incorporate the weighting of each symptom/sign within each symptom cluster. The factor loadings in the confirmatory set fell within 95% confidence intervals (CI) of those in the test set, indicating the stability of the model. The area under the ROC curve for prediction of severity of symptoms with the total score was AUC 0.697 (95% CI 0.593 to 0.802) suggesting reliability of the model.

The RIBI-score model is potentially simple and practical for use by nurses and doctors. This score will require validation in a long-term prospective multi-center study, to prove its reliability for use in the clinical setting.

6.2.3 Cell-Cycle Markers – Use and Application in Clinical Oncology

Multi-parameter analysis of cell-cycle markers has shown a strong relationship between cell-cycle progression and tumour grade, stage and clinical outcome in penile (Kayes et al, 2009), breast (Loddo et al, 2009), and ovarian (Kulkarni AA et al, 2007) cancers. We sought to link expression of cell-cycle phase specific markers in cervical cancer to tumour to clinical outcome, to investigate their potential roles as markers of chemo-radio-sensitivity. Pre-treatment biopsy specimens were obtained for 35 patients with cervical

cancer (stage IB2-IVA) and 12 normal cervix control cases. Each patient had received neo-adjuvant chemotherapy prior to conventional concomitant (chemo)-radiation, as part of a phase II trial conducted at the North London Cancer Network.

Immuno-histochemical staining was performed using a panel of cell-cycle phase markers; Replication Licensing Factors; Mcm2 and Geminin, and the standard proliferation marker Ki67 (clone MIB-1). The expression of each marker was high in all cases of squamous cell carcinoma (SCC) regardless of stage or grade of disease. Our results showed that all cases displayed an aggressive, so-called ' actively-cycling phenotype'. Univariate analysis showed no correlation with level of expression and clinical outcome, unsurprising given the high level of expression of markers in all cases. All the cases of squamous cell carcinomas (n=28) and all one of the adenocarcinomas (n=7) displayed an actively cycling phenotype.

Seven out of the 35 (20%) women experienced significant symptoms of radiation-induced bowel injury (RIBI), with no difference in marker expression or clinical outcome (disease-free survival). Of the 20% (7/35) of patients in this cohort who reported symptoms of RIBI, 3/7 of them had required endoscopic examination with rectal biopsies. There were no differences in cell-cycle expression (or survival) found between these patients and who did not report any symptoms suggestive of RIBI. Of course, conclusions cannot be drawn from such a small sample. The potential however, for markers of proliferation as a surrogate marker for both (chemo)-radiosensitivity and the risk of normal tissue sensitivity warrants further exploration.

6.2.4 Use of cell-cycle markers to define colo-rectal crypt cellularity after radiation treatment and relevance to symptomatology and severity of RIBI.

The patho-physiology of radiation-induced bowel injury (RIBI) is still not well understood. It is unclear why some cancer survivors develop significant symptoms, and it is well accepted that symptoms of RIBI most likely arise as a result of multiple functional, structural and physiological deficiencies related to radiation injury. The aim

of this chapter was to investigate proliferation profiles in colonic crypts of women who presented with bowel symptoms after radiotherapy. I sought to investigate any correlations between the proliferation status in the crypt compartments and presenting symptom 'cluster', severity of symptoms and histological findings.

Sections from colonic biopsy of 56 women investigated for symptoms of RIBI and 17 controls were immunostained with antibodies to Mcm2, Ki67 (clone MIB-1) and Geminin. The percentage of immunopositive epithelial nuclei was determined by calculating a labeling index (LI) for mucosal crypt thirds. I demonstrated reduced expression of all proliferation markers in samples from women with RIBI compared to normal controls ($p<0.05$). This analysis suggests decreased expression of Mcm2, Ki67 and Geminin in the proliferative compartment (basal third) of colonic crypts in women presenting with symptoms of RIBI compared to normal controls; this is in contrast to increased cell cycle entry described in active inflammatory bowel disease (Davies et al, 2003).

The symptom cluster 2; (*diarrhoea, increased bowel frequency, and faecal incontinence*), was associated with the highest expression of Mcm2 and Ki67 in the basal third (proliferation compartment) of crypts. In my analysis in chapter 2, I showed that scoring high for symptoms cluster 3 (*bloating, flatulence, rectal bleeding and per-rectal mucus*) was associated with the most severe symptoms of RIBI at follow-up. It can be presumed although not concluded, that the finding of highest expression of proliferation markers in the basal third of colonic crypts in patients scoring highest for symptom cluster 2 suggests a correlation between proliferation (and hence repair capacity) compared to patients who presented in symptom cluster 3 (and who were more likely to have severe symptoms). These results may suggest some variation in proliferation in mucosal crypt cells depending on patho-physiology.

6.3 Conclusions and Implications for Cancer Survivors

The prevalence of radiation-induced bowel toxicity remains higher than that of Inflammatory Bowel Disease (IBD) and continues to rise (Andreyev et al, 2012). There is much evidence in the literature to suggest gastrointestinal toxicity remains a significant factor in the quality of life of survivors (Andreyev, 2007), yet research into understanding and reducing toxicity is limited.

Evidence from animal models in IBD suggests similar patho-physiology in patients presenting with symptoms of RIBI (Hauer-Jensen et al, 2014). Whilst in IBD, the 'toxic' agent, or 'insult' has yet to be clearly identified, dose-response animal models in radiation make it possible to investigate relationships normal tissue responses after radiation exposure (Hauer-Jensen et al, 1998). It remains difficult to extrapolate results from animal models to the understanding of inter-patient variability in normal tissue responses to radiation. In an experimental study using sections from colonic biopsy and resection specimens of 48 patients with IBD, Davies et al, (2003), described an increase in cell cycle entry as indicated by expression of Mcm2 (and to a lesser extent Ki-67) in the superficial third of colonic glands in active disease compared to inactive disease.

Cell-cycle entry of epithelial cells in the middle and superficial thirds of glands in active IBD has been shown to be consistent with the local release of pro-inflammatory cytokines in active inflammation, together with a direct response to injury of epithelial cells (Rhodes and Campbell, 2002). It is not yet clear whether the observed increase in proliferative activity in IBD is associated with epigenetic and kinetic changes or merely reflects hyper-proliferation associated with active inflammation.

It is difficult given the small numbers in our study to know what conclusions can be drawn and whether it can be extrapolated that the observed decrease in proliferative activity may reflect a sustained loss of repair capacity of the crypt epithelium in patients with RIBI. The site of injury is usually multiple in patients with RIBI (Andreyev, 2007) and sometimes difficult to identify. The management of these patients can be challenging

and this study, I believe raises interesting questions that need to be evaluated in larger, prospective cohorts.

6.4 Clinical Relevance and Future Work

There is a wealth of knowledge to be gained from studies to investigate the patho-physiology of RIBI and thus improve the management of these cancer survivors. Prospectively collected long-term data, using patient and clinician- reported tools is needed, as well as tissue samples from both primary tumour and from multiple sites of bowel from patients presenting with radiation-induced bowel injury.

Following on from this work, the Gynaecological oncology team at University College London Hospital are developing a 'Pelvic Radiation Disease' working group to create structured pathways for managing these patients as well as collecting long-term data.

The currently recruiting INTERLACE STUDY which is investigating the role of neo-adjuvant treatment in patients with locally advanced cervical cancer will be used as a platform to collect prospective toxicity data as well as a bio-bank for both tumour and bowel tissue for women investigated for symptoms of radiation-induced bowel toxicity. This will be a reliable cohort to continue this work at UCLH – the group will be looking to validate the clinical score, whilst collecting data on presenting symptoms, and investigate cell-cycle markers on a larger scale.

Publications

FULL PAPER

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Radiation-induced bowel injury: the impact of radiotherapy on survivorship after treatment for gynaecological cancers

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Background: The number of women surviving cancer who live with symptoms of bowel toxicity affecting their quality of life continues to rise. In this retrospective study, we sought to describe and analyse the presenting clinical features in our cohort, and evaluate possible predictors of severity and chronicity in women with radiation-induced bowel injury after treatment for cervical and endometrial cancers.

Methods: Review of records of 541 women treated within the North London Gynaecological Cancer Network between 2003 and 2010 with radiotherapy with or without chemotherapy for cervical and endometrial cancer identified 152 women who reported significant new bowel symptoms after pelvic radiation.

Results: Factor analysis showed that the 14 most common and important presenting symptoms could be 'clustered' into 3 groups with predictive significance for chronicity and severity of disease. Median follow-up for all patients was 60 months. Univariate analysis showed increasing age, smoking, extended field radiation, cervical cancer treatment and the need for surgical intervention to be significant predictors for severity of ongoing disease at last follow-up. On multivariate analysis, only age, cancer type (cervix) and symptom combinations/'cluster' of (bloating, flatulence, urgency, rectal bleeding and per-rectal mucus) were found to be significant predictors of disease severity. Fifteen (19%) women in the cervical cancer group had radiation-induced bowel injury requiring surgical intervention compared with five (6.7%) in the endometrial cancer group.

Conclusion: Women with cervical cancer are younger and appear to suffer more severe symptoms of late bowel toxicity, whereas women treated for endometrial cancer suffer milder more chronic disease. The impact of radiation-induced bowel injury and the effect on cancer survivorship warrants further research into investigation of predictors of severe late toxicity. There is a need for prospective trials to aid early diagnosis, while identifying the underlying patho-physiological process of the bowel injury.

Do Cell-Cycle Phase-Specific Markers Predict Disease Grade, Stage, and Outcome in Cervical Carcinoma?

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Abstract

AIMS:

Multiparameter analysis of cell cycle markers has shown a strong relationship between cell cycle progression and tumor grade, stage, and clinical outcome in penile, renal, ovarian, and breast cancers. We sought to link expression of cell cycle phase-specific markers in cervical cancer to tumor grade, stage, and clinical outcome to investigate their potential use as prognostic and predictive markers.

METHODS:

Pretreatment biopsy material was obtained from 35 patients with cervical cancer (stage IB2-IVA) and 12 normal cervix control cases. Each patient was treated with neoadjuvant chemotherapy followed by chemoradiation. Immunohistochemical staining was performed using a panel of cell cycle phase markers: replication licensing factors: Mcm2 (minichromosome maintenance 2) and geminin, and the standard proliferation marker Ki67 (clone MIB-1).

RESULTS:

The expression levels of each cell cycle biomarker were very high in all cases of squamous cell carcinoma of the cervix regardless of grade or stage of disease. In our cohort, all cases displayed an aggressive, so-called actively cycling phenotype. Univariate analysis showed that none of the cell cycle biomarkers predicted grade, stage, or clinical outcome.

CONCLUSIONS:

Cell cycle phase-specific markers do not appear to predict disease grade, stage, or outcome in our sample of patients with cervical cancer. This is not surprising, given that the expression of each cell cycle biomarker was very high in all cases. Indeed, all the cases of squamous cell carcinoma of the cervix (n = 28) and all but 1 of the adenocarcinomas (n = 7) in this study displayed an aggressive "actively cycling" phenotype. This predominance of actively cycling tumors is unusual and may reflect the viral etiology underlying the disease. These preliminary findings raise many interesting questions including the prognostic value of disease grade and markers of proliferation in cervical tumors as reliable prognostic indicators. Further work on a larger cohort of patients is warranted.

Paper for Submission:

Radiation-induced Bowel Injury (RIBI); Can Cell-Cycle phase specific markers in colo-rectal mucosal crypt cells predict clinical features and outcome following Pelvic Radiotherapy for Cervical and Endometrial Cancers?

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Abstract

Background: The patho-physiology of Radiation-Induced Bowel Injury (RIBI) is still not well understood. It is unclear why some cancer survivors develop significant symptoms. The aim of this study was to investigate proliferation profiles in colonic crypts of women presenting with symptoms of chronic RIBI and determine correlations with presenting symptom 'cluster', severity and histological findings.

Methods: Sections from colonic biopsy of 56 women investigated for symptoms of RIBI and 17 controls were immunostained with antibodies to Mcm2, Ki67 (clone MIB-1) and Geminin. The percentage of immunopositive epithelial nuclei was determined by calculating a labelling index (LI) for mucosal crypt thirds.

Results: Reduced expression of all proliferation markers was found in samples from RIBI compared to normal controls ($p<0.05$). The symptom cluster of diarrhoea, increased frequency, and faecal incontinence was associated with the highest expression of Mcm2 and Ki67 in the basal third of crypts.

Conclusions: Our analysis suggests decreased expression of Mcm2, Ki67 and Geminin in the proliferative compartment (basal third) of colonic crypts in women presenting with symptoms of RIBI compared to normal controls. This is in contrast to increased cell cycle entry described in active inflammatory bowel disease. Our results also suggest some variation in proliferation in mucosal crypt cells depending on presenting symptom cluster that warrant further investigation.

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